Cellular responses to Cd, Pb and Zn in shrews (*Myosorex varius* and *Crocidura flavescens*) and BALB/c mice

By

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously, in its entire or in part, submitted it at any other university for a degree.

Abstract

Shrews are excellent bioindicators of environmental heavy metal pollution. This is due to their diets and the high rate of consumption at which these small mammals consume their prey. Shrews feed mostly on earthworms, and earthworms accumulate high levels of heavy metals in their bodies. Biomarkers measure responses to environmental pollution in the bodies of exposed organisms. Cellular biomarkers give an indication of responses to pollutants at levels of pollutant exposure that are not yet lethal to the organism.

This study was prompted by concern that the number of shrews in the Stellenbosch region was declining. For the present study, shrews (*Myosorex varius* and *Crocidura flavescens*) from the Stellenbosch region were sampled. The levels of Cd and Pb in their bodies were analysed in order to ascertain whether or not these two heavy metals, which occur in the environment as a result of various anthropogenic activities, are present in the food chain of the shrews. In the laboratory, shrews were fed live control and metal exposed earthworms from laboratory cultures. The earthworms had been exposed to either Cd or Pb in these cultures over a number of generations. This was performed to determine whether the presence of the metals in the body of the shrews, had resulted from metal accumulation from the earthworms which were fed to the shrews. Cellular biomarkers were used to determine the cellular response to the metals and membrane integrity and DNA integrity were investigated. This was done by exposing the cells of the shrews and of BALB/c mice to metal salt solutions of Cd, Pb and Zn *in vitro*. Membrane integrity was tested by cell leaching techniques viz. the LDH assay and the trypan blue assay. DNA integrity was determined by using the comet assay as a biomarker of exposure.

The results of the study show that the shrews in the immediate Stellenbosch region are exposed to lower levels of Cd and even lower levels of Pb than shrews from a site 16km out of the town of Stellenbosch. The cellular responses induced by metal exposure of laboratory mice show that the metals are indeed cytotoxic and genotoxic to the cells of small mammals. Cd and Pb were found to be more cytotoxic than Zn, while Zn was more genotoxic than either Cd or Pb.

Opsomming

Skeerbekke is uitstekende bioindikatore van swaarmetaalbesoedeling in die omgewing. Dit is as gevolg van hul dieët en die hoë koers waarteen hierdie klein soogdiere hul prooi verorber. Skeerbekke voed meestal op erdwurms, en erdwurms akkumuleer hoë vlakke van swaarmetale in hul liggaam. Biomerkers meet response as gevolg van omgewingsbesoedeling in die liggame van blootgestelde organismes. Sellulêre biomerkers gee 'n aanduiding van response op besoedelingstowwe by vlakke wat nog nie dodelik is vir die organisme nie.

Die studie is aangespoor deur die bekommernis dat die getal van skeerbekkies in Stellenbosch besig is om te daal. In die studie is skeerbekke (*Myosorex varius* en *Crocidura flavescens*) van die Stellenbosch omgewing gevang en die vlakke van Cd en Pb in hul liggame gemeet om vas te stel of die twee swaarmetale, wat in die omgewing beland as gevolg van verskeie antropogeniese aktiwiteite, teenwoordig is in die voedselketting van die skeerbekke. Die skeerbekke is in die laboratorium met lewende kontrole en metaalblootgestelde erdwurms uit kulture in die laboratorium gevoer. Die erdwurms is aan Cd en Pb oor baie generasies blootgestel in hierdie kulture. Dit is gedoen om vas te stel of die teenwoordigheid van die metale in die liggame van die skeerbekke 'n oorsaak is van metaal-akkumuleering as gevolg van die erdwurms waarmee hulle gevoer is. Sellulêre biomerkers is gebruik om sellulêre respons op die metale op die membraanintegriteit en DNA-integriteit vas te stel. Dit is gedoen deur die selle van skeerbekke so wel as BALB/c muise *in vitro* bloot te stel aan metaalsoutoplossings van Cd, Pb en Zn. Membraanintegriteit is getoets deur sel-membraan permeabiliteit tegnieke nl. die LDH en tripaan blou toetse. DNA-integriteit is getoets deur die komeettoets te gebruik.

Die resultate van die studie dui daarop dat die skeerbekke van Stellenbosch aan laer vlakke van Cd, en nóg laer vlakke van Pb blootgestel is as die skeerbekke van 'n perseel 16km buite die dorp. Die sellulêre response geïndusseer deur blootstelling aan metale by die laboratoriummuise het aangetoon dat die metale inderdaad skadelik is vir die selmembraan en die DNA van die selle van die muise. Cd en Pb is meer toksies vir die selmembraan as Zn, terwyl Zn weer meer toksies is vir die DNA-integriteit as Cd en Pb.

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Chapter 1

General Introduction

Concern that the number of shrews in the Stellenbosch region, in the Western Cape was declining, was the impetus for this study. One reason postulated was that the number of shrews could be declining because of increasing toxic metals in the environment to which they are exposed. A number of authors have proposed that the vulnerability of the shrews to toxic metals is a result of their diet (Andrews, Johnson & Cooke 1984; Ma 1989; Ma, Denneman & Faber 1991) and the high consumption rates (due to their high metabolism) at which these small insectivorous mammals consume invertebrates. Especially earthworms are consumed and this increases the exposure that the shrews have to toxic metals in comparison to herbivores or carnivores (Andrews *et al.* 1984; Ma 1987; Ma 1989; Ma *et al.* 1991). Earthworms make up a large part of the diet of shrews (Churchfield 1990) and are known to accumulate toxic metals (Ireland 1979; Ma *et al.* 1991). Heavy metal accumulation in the bodies of exposed mammals may exert adverse health effects when certain levels of metals in target organs, identified for specific metal accumulation are reached. Johnson & Roberts (1978), while studying the distribution of Cadmium (Cd), Lead (Pb) and Zinc (Zn) in small mammals that inhabited an industrial area found that renal disorders resulted in shrews exposed to Cd and Pb. Ma *et al.* (1991) reported levels of Cd and Pb in the kidney, the critical organ for metal accumulation that would be indicative of adverse renal effects if exceeded.

In the present study, shrews were used as bioindicators of Cd and Pb exposure in their natural environment. Shrews are suitable bioindicators of environmental exposure (Ma 1989; Pankakoski, Koivisto, Hyvärinen, Terhivuo & Tähkä 1994b). According to Van Gestel & Van Brummelen (1996), bioindicators are organisms that give information on the environmental condition of their habitat by their presence, absence or behaviour in the habitat. Ma (1989) studied small mammals as bioindicators of Pb exposure in the environment. The results of that study showed that the level of Pb in the kidney, liver and bone of the small mammals he studied, were elevated in comparison to the levels of Pb found in the organs of small mammals from the control site, indicating the bioavailability of Pb to small mammals from the food chain.

Body concentrations indicate the ability of metals to enter the food chain (Ma 1989), but body loads alone can not be used to accurately predict hazard to small mammals since one does not know to what degree the levels of body load and the levels of metals in soil compare. Shore (1995) states that one needs robust data in order to predict hazard accurately. Ma *et al.* (1991) states that hazard assessment of bioavailable metals can be derived by comparing oral metal intake rates and organ metal load, in association with critical levels and sublethal exposure symptoms.

According to Shugart, Adams, Jiminez, Talmage & McCarthy (1989a), the detection and estimation of risk in wild animals is possible by monitoring biological endpoints. The authors suggest three criteria that biological endpoints need to indicate. These include quantifying exposure by measuring chemical interaction with critical molecule targets; measuring alteration in specific sensitive and critical physiological and biochemical processes and monitoring early expression of cellular dysfunction. These authors view the organism as an integrator of exposure accounts from abiotic and physiological factors that change the dose of the toxicant that is taken up from the environment. Biomarkers are used to detect the responses of the organism to environmental pollution and Van Gestel & Van Brummelen (1996) define the term biomarker, as the biological response to chemical exposure, that is measured inside the body of the bioindicator or in it's products. The organism needs to be a sensitive biological indicator that detects exposure effects early in time because of the latent period that exists between exposure and detectable irreversible damage. Van Gestel & Van Brummelen (1996) stress the importance of the association of biomarkers with an early warning system of pollution induced stress. This association is to be seen either as the detection of pollutant early in time, i.e. soon after the release/emission of it from its source; or as detection at concentrations below that which cause irreversible damage. The detection of a pollutant early in time, warns that effects may develop with time; while detection of pollutants at levels which do not result in irreversible damage, does not indicate that severe effects will develop in time.

Cellular responses to pollutants that are induced by cell damage/injury provide a rapid and highly sensitive indication of environmental impact on the cell (Moore 1985). It should be possible to observe alterations to the structural and functional organisation in individual/groups of target cells before the whole animal levels of physiological processes are affected and population levels are changed. Cellular biomarkers may or may not be specific in terms of pollutant response (Walker, Hopkin, Sibly & Peakall 1996). Neutral red retention is a non-specific cellular biomarker that measures lysosomal membrane integrity as a response to chemical exposure (Svendsen & Weeks 1995). The inhibition of ALAD by Pb and the inhibition of acetlycholinesterase by organophosphorous and carbamate pesticides are specific cellular biomarkers (Walker *et al.* 1996).

Cadmium is a relatively rare metal (Hiatt & Huff 1975; Stoeppler 1992; Wren, Harris & Harttrup 1995) that is found in the presence of ores of other metals e.g. Zn (Hiatt & Huff 1975; Richardson 1993; Wren *et al.* 1995). It is a by-product of lead, cadmium, copper, nickel and zinc smelting nd the main uses are in electroplating, pigment manufacture, batteries (Richardson 1993; Wren *et al.* 1995) and plastic stabilisers (Hiatt & Huff 1975). Small amounts of Cd are also used in the production of pesticides (Hiatt & Huff 1975; Richardson 1993). Significant amounts of Cd are also found in phosphate fertilisers (Samarawickrama 1983; Cooke & Johnson 1996) and fossil fuels (Wren *et al* 1995; Cooke & Johnson 1996). According to Cooke & Johnson (1996), Cd is one of the most mobile toxic metals. It is transferred along most terrestrial food chains from plants to animals and the potential for its transference is greater than that of either Pb or Zn.

Cadmium has no known biological function and its toxicity may originate through exposure via respiration or ingestion (Cooke & Johnson 1996). Cadmium is sequestered in metallothionein complexes that contain a high proportion of sulphur binding sites that bind with Cd rendering it unavailable for interaction with intracellular receptor sites (Wren *et al* 1995; Cooke & Johnson 1996). The main accumulation sites for Cd are the kidney and liver (Wren *et al* 1995).

Lead is the most ubiquitous toxic metal and all known effects of this metal on biological systems are deleterious (Bryce-Smith & Stephens 1983; Pain 1995). It is an important constituent of more than 200 minerals and occurs in both organic and inorganic forms (Pain 1995). Pb is also found in Pb-containing compounds like plastics, putty, paint, batteries and ceramic glazes (Haley 1968). The major natural source of lead in soils results from the weathering and mineralisation of parent material. The uptake of Pb by plants is limited by the low availability of Pb in soils and the intake of Pb in terrestrial vertebrates is mainly via inhalation and ingestion (Pain 1995). Lead exposure in mammals is specifically indicated by the structural changes in the kidneys of Pb-exposed mammals (Ma 1996).

Zn has a reputation for being one of the less toxic metals (Harris 1991). Large amounts of Zn can be given orally without severely damaging tissue or impairing biochemical function (Harris 1983; Miller 1983) and high concentrations of Zn probably decrease cadmium toxicity in wildlife (Wren *et al.* 1995). It is a nutritionally essential metal in mammalian metabolism (Richardson & Gangolli 1994b; Ganong 1995b) and in the environment it is found in association with Cd (Hiatt & Huff 1975; Richardson 1993). It is used as plating for cathodic protection against corrosion of steel, in alloys and in galvanic cells and storage batteries (Richardson & Gangolli 1994b).

According to Johnson & Roberts (1978), the biological impact on mammals to Pb or Cd exposure is normally reflected by renal disorders and histopathological changes at higher levels of intoxication. The toxicology of Zn is less clearly defined because of the various homeostatic mechanisms that control Zn absorption (Bertholf 1986).

The aims of the present study were:

i. To determine heavy metal concentrations of Cd and Pb in the soil at two different sampling plots in the Stellenbosch region; one in the town and one in an agricultural area;

ii. To collect shrews from both areas and to determine the body concentrations of Cd and Pb in these animals;

iii. To keep shrews in the laboratory under controlled conditions and to feed them with control and metal-contaminated food (minced meat and earthworms) to determine transference of the metals from invertebrates to mammals;

iv. To use cells from the spleens of shrews and to investigate the effects of the heavy metals (Cd, Pb and Zn) on the cellular level;

v. To compare and verify results of the cellular tests on shrews with a laboratory bred mouse strain (BALB/c);

vi. To do *in vitro* exposures of shrews and mice cells with the heavy metal salts ($CdCl_2$, $PbCl_2$ and $ZnCl_2$) and to measure the effects thereof on membrane integrity (LDH and Trypan blue assays as well as DNA synthesis with $^{[3]}H$ Thymidine incorporation) and DNA integrity (comet/single cell gel electrophoresis assay).

Shrew splenocytes were used in order to determine whether or not Cd, Pb and Zn exhibited cellular cytotoxicity since the results showed that the shrews were exposed to high levels of Cd and Pb in the environment. Splenocytes were used because they represent the cells of the immune system of terrestrial vertebrates and many of the studies done on the cytotoxic effects of Cd and Pb were performed on immune system cells. It is not known if, and how, the different shrew cell types react to metals. The cytotoxic effects of Zn to shrew and mouse splenocytes and to mouse whole blood cells was investigated since the literature showed that Cd and Zn are found in nature in association with one another. It is known that Zn can reverse the effects of Cd and that Zn stabilises plasma membranes. BALB/c mice were used because of the difficulty experienced in trapping and keeping large enough numbers of shrews. Shrews are also very sensitive and develop high stress levels when handled. They may even die as a result of handling. The mice were chosen because they are a known inbred strain and have been bred as mammalian models for many immunological and cytological studies. BALB/c mice are also bred to be docile and especially the females are easy to handle.

In Chapter 2, the general materials and methods, not described specifically in the following chapters, are given. Chapter 3 deals with the transfer of Cd and Pb to shrews from the environment. In Chapter 4, the *in vitro* cellular studies done on both shrew and BALB/c mouse cells are reported on. Chapter 5 deals with the genotoxic effects of Cd, Pb and Zn to mouse whole blood cells. The comet assay was used to measure the effects of Cd, Pb and Zn on the genetic material of the mouse cells.

The conclusion of this study is given in Chapter 6. Two Appendices are added that include the recipes for the solutions that were used in the comet assay as well as the raw data of the experiments performed during this study that have not been included in the various chapters.

Chapter 2

General Materials and Methods

2.1 Test animals

2.1.1 Shrews

These animals are small, short-legged mouse-like mammals with long pointed snouts, and hairy tails, and short, dense, usually dark brown fur. They have small eyes, usually hidden in fur and very small, rounded often quite inconspicuous ears (Smithers 1983; MacDonald 1985; Churchfield 1990). Shrews are robust creatures with voracious appetites and high metabolic rates. They have been known to man from the earliest ages as can be deduced by mention made of them in old folklore tales from the Ancient Romans to William Shakespeare.

Shrews consume a large variety of invertebrates e.g. beetles, termites, spiders, insect larvae and earthworms (Churchfield 1990; Dickman 1995). According to MacDonald (1985), they consume large amounts of food for their size, and their metabolic rate is higher than the metabolic rate of rats of comparable size. Digestion in shrews is very rapid; the gut is usually empty after only 3 hours. Their frequent feeding ensures that they carry reserves for only 1-2 hours. They are therefore active throughout the day and night. In captivity though, shrews eat only one to two thirds of their body weight in meat (Smithers 1983).

They are geographically widely distributed (MacDonald 1985). They occur in all major land areas except Australia, New Zealand, Tasmania, Antarctica, Iceland, Greenland, the Arctic islands, Ungava, the West Indies and some of the Pacific islands (Churchfield 1990).

According to Smithers (1983), the African shrews (family Soricidae) first appeared on the continent during the Miocene (26 million years ago). In southern Africa, there are four genera and 15 species of shrews. The four distinct lineages of shrews in southern Africa are the forest shrew (genus *Myosorex*); the climbing shrew (genus *Sylvisorex*); the musk shrews (genus *Crocidura)* and the dwarf shrews (genus *Suncus*). All African species of shrew belong to the subfamily Crocidurinae. These are the white-toothed shrews and they share a unique trait: caravanning. This can be seen from about two to three weeks before weaning, when the young are mature enough to leave the nest. They form a line and each animal grips the rump of the one in front of it and the foremost grips that of the mother. The grip is so tenacious that

if one were to pick the mother up, the whole caravan would be lifted off the ground, intact (MacDonald 1985).

Shrews occupy mostly moist, terrestrial environments with a thick vegetation cover and where a large number of invertebrates are present. Their small size enables them to make use of thermally protected microhabitats. They are also found close to aquatic habitats. A few shrews live around human dwellings and some have even been caught inside houses. Most can climb and burrow a little but they tend to opt for ground surfaces, hiding in grass nests or under logs. They also occupy and modify underground tunnels and crevices created by other small mammals (Churchfield 1990).

Both sexes possess musk glands on their flanks and between their front and hind feet. The secretions of these glands make the shrews unpalatable to most carnivores (MacDonald 1985; Churchfield 1990). The glands are activated by sex hormones and the main function is to lead the two sexes to one another (Smithers 1983).

2.1.1.1 *Myosorex varius*

2.1.1.1.1 Classification

Phylum: Chordata
Subphylum: Vertebrata
Class: Mammalia
Subclass: Theria
Order: Insectivora
Family: Soricidae
Genus: *Myosorex* (Gray, 1837)
Species: *varius* (Smuts, 1832)

2.1.1.1.2 Morphology, occurrence and habits

Myosorex varius, is also known as the Forest shrew. This species of shrew is endemic to southern Africa and it is also the most widely distributed forest shrew (Figure 2.1). It is found in a great variety of vegetation types i.e., primary forest and montane grassland to vegetation that is largely associated with permanent water bodies on the Highveld (Dippenaar 1997). In the Western Cape, it occurs along the coastal areas in moist, densely vegetated areas (Smithers 1986).

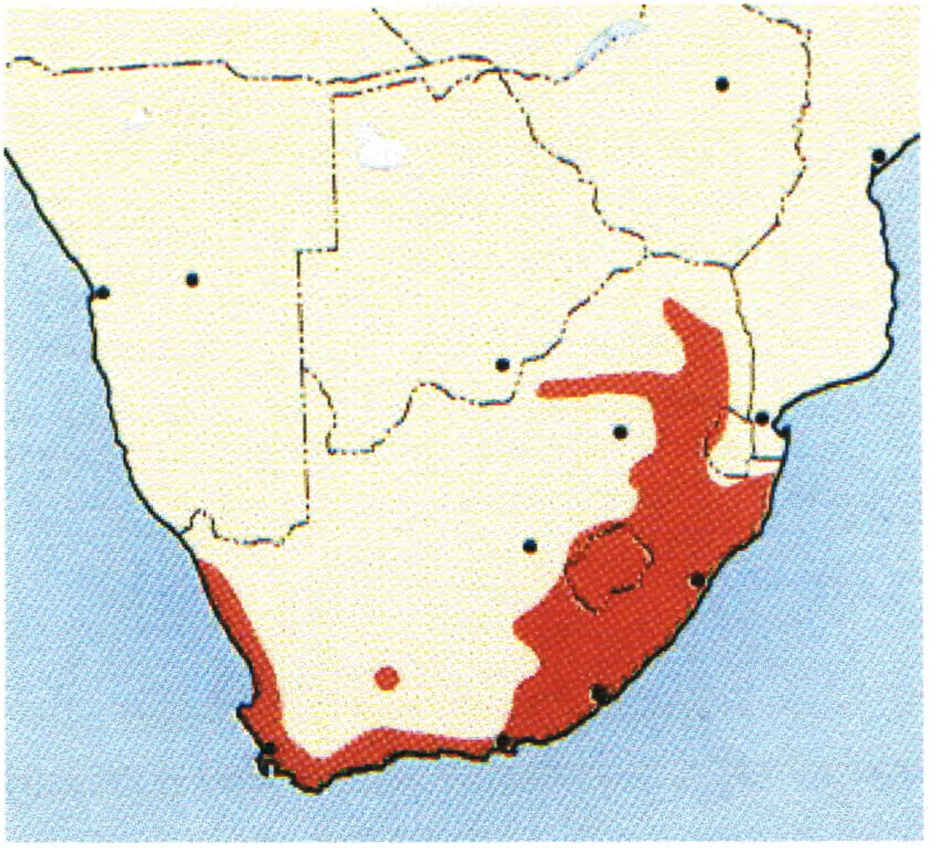

Figure 2.1 Distribution of *Myosorex varius* in southern Africa (adapted from Dippenaar 1997)

It is a predominantly brown shrew (Figure 2.2). The underparts of its body being lighter brown than the top. The head and body length of an adult *M. varius* shrew, are approximately 83mm. Tail length comprises 52% of the head and body length and is also brown in colour. The average adult shrew weighs 12g (Dippenaar 1997). The hands, feet and claws are a pale brown colour (Roberts 1951).

Figure 2.2 The Forest shrew (*Myosorex varius)* (Jackson 1997)

Myosorex varius is a nocturnal animal. It has peaks of activity at dusk and its activity declines at dawn (Smithers 1983; Dippenaar 1997). The forest shrew burrows under rocks and uses pre-existing burrows as nests. These are then lined with soft debris (Smithers 1983).

According to Dippenaar (1997) *M. varius* is an opportunistic feeder. It feeds on a wide variety of invertebrates, i.e., beetles, grasshoppers, termites, moths, spiders, millipedes and earthworms. They drink water by throwing their heads backward and letting the water trickle down their throats (Smithers 1983; MacDonald 1985; Churchfield 1990).

Vocalisations made by the shrews include short, high-pitched squeaks when they are startled. The shrews during aggressive encounters (Smithers 1983; Dippenaar 1997) make repeated vocalisations with frequently interspersed drawn-out "chirrs".

The breeding season lasts from September to March and the average litter size is usually three, but may vary between two and five. Maternal care is well developed and for the first five to six days, nipple clinging is obligate. This is followed by clustering and then caravanning. Known predators of the forest shrew are barn owls, water mongooses, the African weasel and the striped polecat (Dippenaar 1997).

2.1.1.2 *Crocidura flavescens*

2.1.1.2.1 Classification

Phylum: Chordata
Subphylum: Vertebrata
Class: Mammalia
Subclass: Theria
Order: Insectivora
Family: Soricidae
Genus: *Crocidura* (Wagner, 1832)
Species: *flavescens* (Geoffroy, 1827)

2.1.1.2.2 Morphology, occurrence and habits

Crocidura flavescens, is also known as the Greater Red Musk shrew and it is the largest shrew in southern Africa (Smithers 1983). It is reddish brown in colour and has a distinct musky smell as its name suggests. This shrew is endemic to Southern Africa. It is found in vegetation types such as fynbos, temperate and subtropical grasslands and coastal forests. It is also found in woodland and savannah biomes (Dippenaar 1997) (Figure 2.3). In

the Western Cape it occurs along the coastal areas with a rainfall in excess of 500mm per annum (Smithers 1986).

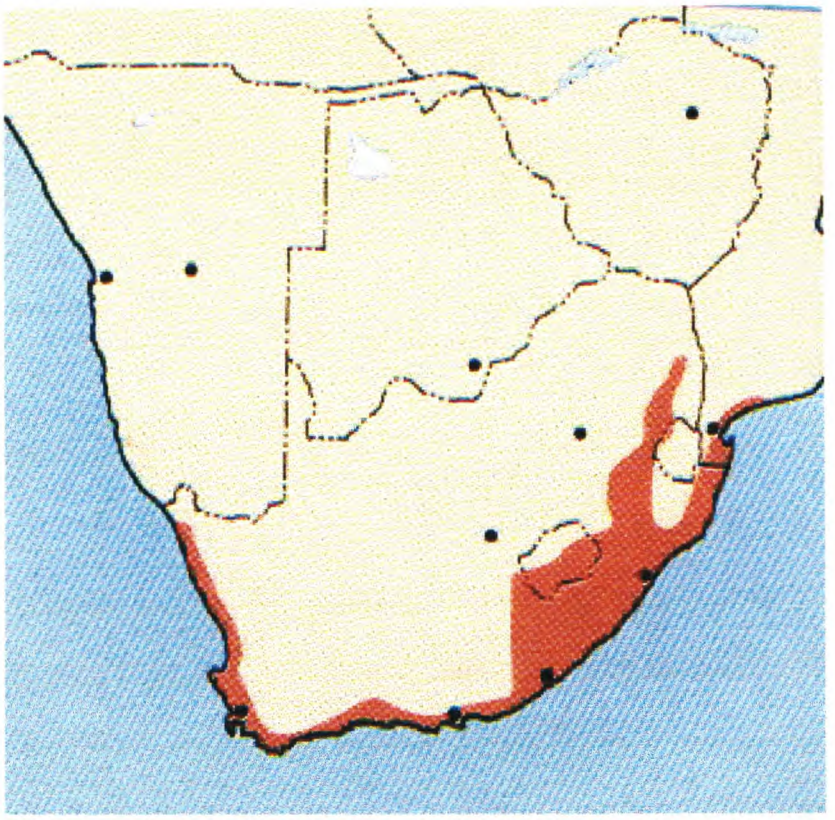

Figure 2.3 Distribution of *Crocidura flavescens* in southern Africa (adapted from Dippenaar 1997)

A degree of colour variation does occur within this species: in the Western Cape it is reddish brown with brownish grey underparts; in Mpumalanga it is pale fawn with whitish underparts (Figure 2.4) (Smithers 1986). Its head and body make up approximately 105mm of the adult body length. The average adult *C. flavescens* shrew weighs 24g (Dippenaar 1997).

Figure 2.4 The Greater red musk shrew (*Crocidura flavescens)* (Baxter 1996)

Dippenaar (1997) describes this species as pugnacious and predominantly nocturnal with peaks of activity occurring at dusk and dawn.

Crocidura flavescens is an opportunistic feeder – it is both insectivorous and carnivorous and has been known to scavenge (Dippenaar 1997). In captivity, these shrews are both carnivorous and cannibalistic (Smithers 1983).

Vocalisations are loud and the shrews emit strident squeaks when they are frightened. The lateral, chin and ano-genital glands are used for scent marking and faecal piles are strongly scented.

The breeding season lasts from August to April. Usually, the litter consists of four young but this number may vary from one to seven. Maternal care is well developed and for the first five to six days, nipple clinging is obligate. This is followed by clustering and then caravanning (Dippenaar 1997).

2.1.2 BALB/c mice

2.1.2.1 Origin and history

According to Les (1995), Potter (1985) has reviewed the history and characteristics of this inbred mouse strain. According to him, Dr Halsey J. Bagg of Memorial Hospital, New York, obtained albino (white coat with pink eyes) breeding stock from a mouse dealer in Ohio in 1913. Dr's Little and MacDowell obtained mice from Dr Bagg and bred them too. In 1913, H. J. Muller, from the University of Texas, acquired mice from MacDowell, and in that same year, George Snell acquired a breeding colony from Muller. In 1935, Snell moved to the Jackson Laboratory in Bar Harbour and took the Bagg albinos with him.

Snell added the "c" to the strain name in that same year. The "c" indicates that the genotype at the "colour" locus is "c/c". The strain name was therefor established as BALB/c. Originally, the name was written "Balb/c", but action by the Committee on Standardised Nomenclature for Inbred Strains of Mice led to the capitalisation of the name, written "BALB/c."

According to Vogt, Swartz and Massey (1993), mating closely related animals (for example, parent and offspring, full brother and sister or half brother and sister) is inbreeding. Technically it is defined as the mating of animals more closely related than the average relationship within the breed or population concerned. For practical purposes, if two mated individuals have no common ancestor within the last five or six generations, their progeny would be considered outbreeds.

The primary genetic consequence of inbreeding is to increase the frequency of pairing of similar genes. All genetic and phenotypic changes associated with the practice of inbreeding stem from this one primary consequence. Inbreeding is a very useful tool in the field of animal breeding. It enables the breeder to uncover and eliminate harmful recessive genes within the population. It is also desirable in the development of distinct family lines. Seed stock and commercial producers have successfully used linebreeding to maintain a degree of genetic relationship in their animals to some outstanding ancestor or ancestors.

BALB/c mice were bred as model organisms for biomedical research. The strain is particularly well known for the production of plasmacytomas on injection with mineral oil. These tumours form the basis for the production of monoclonal antibodies. The mice are also used as hosts for transplantable tumours.

2.1.2.2 Classification

Phylum:	Chordata
Subphylum:	Vertebrata
Class:	Mammalia
Subclass:	Theria
Order:	Rodentia
Family:	Muridae
Genus:	*Mus*
Strain:	BALB/c

2.1.2.3 Morphology and habits

BALB/c mice are small, white-coated albinos with pink eyes, hands, feet and tails. They are non-aggressive but males do exhibit fierce male-male aggression when placed together in one cage. They exhibit good breeding performance and generally have a litter size that varies between six and nine young. The life span of females is generally 20 months while males can be expected to have a life span of only 13 months.

2.2 Heavy Metals

2.2.1 Cadmium

Cadmium is the 67th most abundant element in the earth's crust (Stoeppler 1992). It is a soft, silver-white metal with a faint blue tinge that is usually found as impurities in ores of other metals, e.g., Zn (Wren *et al.* 1995).

It was only recently discovered in 1817, and its industrial use was relatively minor till about 50 years ago. Since then, due to its non-corrosive properties, it is being used extensively in electroplating and galvanising. It is also used as a colour pigment in paints and plastic and as a cathode material for nickel-cadmium batteries. Cadmium is also a by-product of lead, cadmium, copper, nickel and zinc smelting and it is also used in the manufacture of combustible fuels (Wren *et al.* 1995)

The deposition of atmospheric Cd and Cd-contaminated fertilisers and irrigation water are the main factors contributing to the presence of Cd in the soil. According to Wren *et al.* (1995), Anderson and Hahlin (1981) have suggested that another potential source of Cd toxicity is the use of commercial sludge to fertilise agricultural fields. This sludge may contain up to 1500 mg $Cd.kg^{-1}$ of dry material.

In vertebrates, Cd is transported in the blood bound to metallothionein. The main accumulation sites are the kidney, liver and intestinal mucosa (Nath, Prasad, Palinal & Chopra 1984).

2.2.2 Lead

Lead is the most ubiquitous toxic metal. It is nonessential and highly toxic. All known effects of Pb on biological systems are deleterious (Pain 1995).

It rarely occurs naturally in its elemental state but does occur as an important constituent of more than 200 minerals. The average concentration of Pb in the earth's crust is 1.6g $Pb./100kg^{-1}$ of soil. Small amounts of Pb are emitted into the environment as a result of naturally occurring processes e.g., weathering of rocks, igneous activity and radioactive decay (Pain 1995).

Lead is easy to mine and has been used by man throughout antiquity. More than 7000 years ago, the Ancient Egyptians used Pb in the manufacture of weights, anchors, cooking utensils, piping and pottery glaze. In Ancient Rome, the solubilisation of Pb from cooking pots, used for preparing grape syrup for wine and the preservation of fruit by the aristocracy, has been suggested as the reason for the decline of the Roman empire (Pain 1995).

Present day anthropogenic Pb emissions lead to soil and water Pb concentrations that are up to several orders of magnitude higher than estimated natural concentrations (Pain 1995). The major sources of environmental Pb contamination results from the commercial uses of Pb containing compounds like plastics, putty, paint, batteries and ceramic glazes. The airborne effluents of zinc and nickel smelters also contribute to environmental Pb contamination. The greatest threat from Pb in the environment originates from pollution by anthropogenic organolead compounds, especially the use of tetraalkylleads (R_4Pb) in petrol. Such compounds have been added to petrol since 1923 to improve octane ratings for fuels used in high compression internal combustion engines (Pain 1995).

Lead distribution in water is dependent on the chemical form of Pb. In the soil, dry and wet deposition of atmospheric Pb occurs. The disposal of sewage sludge onto agricultural lands is also a source of Pb contamination. Lead is relatively immobile when in soil. Ocean beds are known as "sinks" for anthropogenically emitted lead. (Pain 1995).

The intake of Pb in terrestrial vertebrates is mainly via inhalation and ingestion. Dermal absorption of Pb is minimal. After absorption and transport through the bloodstream, Pb is accumulated primarily in the kidneys and liver, and deposited in the bone (Pain 1995). The major risk to Pb toxicity is to the nervous system.

2.2.3 Zinc

Zinc is the 23rd most abundant element in the earth's crust - 0.02% by weight. It is a nutritionally essential metal in mammalian metabolism and deficiency results in severe health consequences. Zinc is required for normal leukocyte development and functioning in concentrations that is not toxic to cells (Koropatnick & Zalups 1997). Zinc has been shown to stabilise plasma and lysosomal membranes (Chvapil 1973; Bettger & O'Dell 1981) and animal studies have shown that Zn can at least partially prevent some known toxic effects of Cd (Nath *et al.* 1984; Harris 1991; Endo, Kimura, Hatakeyama, Takada & Sakata 1997).

It is used as plating for cathodic protection against corrosion of steel, in alloys and in galvanic cells and storage batteries (Richardson & Gangolli 1994b).

Excessive exposure to Zn is relatively uncommon and dependent upon heavy exposure. Zinc is transported in the plasma and is primarily bound to albumin while small amounts may also be bound to globulin fractions and amino acids. Zinc accumulation is homeostatically modulated by mechanisms that act principally on absorption and liver levels (Bertholf 1986).

2.3 Experimental Materials and Methods

2.3.1 Trapping of shrews

From March 1999 to February 2000, shrews of the species *M. varius* and *C. flavescens* were caught in the Stellenbosch and Raithby areas. Traps were also put out in Franschoek at Delta Meer, a densely vegetated area situated next to the Berg River. No shrews were caught here. Two Verreaux's mice (*Myomyscus verreauxii)* were caught and released again. A permit that allowed the capture of a limited number of wild animals (shrews and mice) by means of prohibited hunting methods and a permit to keep said wild animals in captivity was obtained from the Cape Nature Conservation.

Twelve shrews of the species *M. varius* and one *C. flavescens* shrew were caught. Eight of these shrews were female and four were male. The shrews were sexed internally after they had either succumbed or were killed. The aggressive nature of the shrews made it impossible for them to be handled and thus sexed while they were alive.

Sherman traps were used to trap the shrews. Sherman traps are rectangular, lightweight, collapsible aluminium traps (Figure 2.5). The door of the trap is held open by a clip. This clip is attached by a spring to a flat panel on the floor of the trap. The weight of the animal triggers the mechanism to shut the door of the trap when the animal walks over the panel. The traps are adjustable in terms of the weight required to trap the animal (Churchfield 1990).

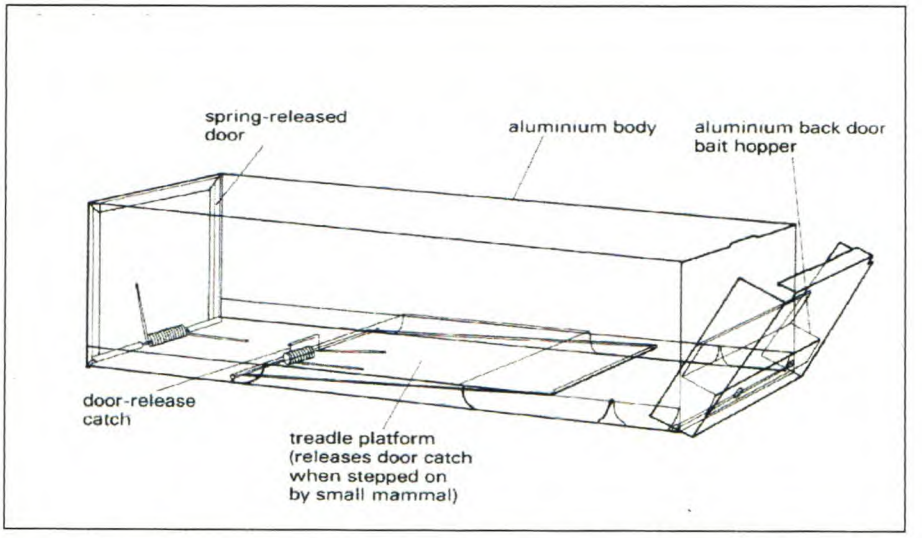

Figure 2.5 A Sherman trap (adapted from Churchfield 1990)

The traps were baited with a mixture of rolled oats, peanut butter, sunflower oil and caramel syrup. The bait mixture had a solid, sticky consistency. A small amount of bait, approximately half the size of a table-tennis ball, was placed inside the trap nearest the closed end of the trap. Care was taken not to let the bait interfere with the shutting mechanism of the trap.

The baited traps were set out in the late afternoon between 16h00 and 18h00 and were then checked again the following morning between 05h30 and 07h00. The traps were always placed under thick vegetation cover so as to hide it from inquisitive people who might disturb the traps and from dogs that might try and eat the trapped shrews. One trap in Franschoek was trampled and crushed by a dog. Sherman traps are not waterproof and are also good conductors of heat. In cold wet weather, the traps were often damp and cold inside. Extra vegetation was then used to cover the top of the traps to keep the inside dry and warm.

2.3.2 Animal Rearing and Exposure

Trapped shrews were immediately brought to the laboratory where they were weighed in empty glass jars on an electronic balance. Shrews were kept individually in appropriate plastic cages (20cm x 12cm x 15cm) with wire-mesh tops. Bedding (woodcarvings) was put inside the cages (to a depth of ± 4cm). Clean glass jars, that were spraypainted black with a commercially available spraypaint, were provided for the shrews as shelter. Cotton wool was put inside the glass jars as extra bedding for the shrews.

The room in which the shrews were kept was climate controlled and the temperature was kept constant at 25°C. Moisture was not controlled for and may have fluctuated during the time that the shrews were kept in the room (total time period equals two months and nine days). The shrews were on a normal day night cycle.

Cadmium and Pb loads were determined in the body of 10 shrews. Eight of the shrews were from Raithby and two were from Stellenbosch. All but one of the five exposed shrews and the single *C. flavescens* shrew was allowed to acclimatise for two days. The other shrew was left to acclimatise for six days. During the acclimatisation period, the shrews were fed the lean beef mince mixture, live earthworms from an uncontaminated culture and had freely available drinking water.

After this period, the shrews were fed the appropriate metal contaminated earthworms (*Eisenia fetida)* from the contaminated cultures in the laboratory. The Cd-exposed group of shrews was fed live earthworms from a culture that was contaminated with sublethal levels of cadmium. The Pb-exposed group of shrews was fed live earthworms from the earthworm

culture that was contaminated with sublethal levels of Pb. The earthworms used during the experiments were from a culture that had been in the contaminated substrates for many generations. The field control group as well as the single laboratory control shrew (*C. flavescens)* was fed uncontaminated earthworms from the control cultures in the lab. The shrews were fed, in addition to the live earthworms from the various cultures, three to five grams of extra-lean minced beef meat which was mixed with ± half a teaspoon of Pronutro and a knife point of Bovril. A vitamin supplement that contained various herbal extracts (the precise formulation is not known) and which was specially prepared and is commercially available was also given to the shrews (one drop over the mince mixture). The shrews (one male and female *M. varius),* used to obtain the spleen cells for the splenocyte cultures were both fed live control earthworms and also had drinking water freely available.

The BALB/c mice that were obtained from the Tygerberg Animal Unit at the University of Stellenbosch Medical School were kept in a climate-controlled room where the ambient temperature was maintained at ± 25°C with a normal day night cycle. Humidity was not controlled for. The mice were kept in plastic cages (20cm x 12cm x 15cm) with wire mesh tops. The cages were lined with woodcarvings as bedding and cleaned once a week. The mice were fed dry cat pellets (either Pampers of Catmor) and had freely available drinking water.

2.3.3 Metal Solutions

Ten millilitres of 10mM $CdCl_2$, $PbCl_2$ and $ZnCl_2$ metal salt stock solutions were made up, with distilled water, sterilely in a laminar flow cabinet (Bino Instrumentation). Metal chloride solutions were used because the chlorides are more stabile and less toxic than the sulphates and nitrates. To obtain the concentration of the metal salts to be used in the *in vitro* and comet assay experiments, metal stock solutions were diluted with heparinised Roswell Park Memorial Institute (RPMI) 1640 medium.

2.3.4 Cell Culture Methods

2.3.4.1 Whole Blood Cultures

To obtain blood, a female BALB/c mouse was put into a 50ml Sterilin test tube that had a hole punched into the top, so that the tail of the mouse could be drawn through it. Female BALB/c mice were chosen for the experiments because they are less aggressive and therefore easier to handle than the male BALB/c mice. The bottom end of the test tube had holes punched into it so that the mouse could breathe. The mouse's tail was wiped with 70%

alcohol to sterilise the surface of the tail. A #11 Carbon steel sterile surgical blade was sterilised with 70% alcohol as well. With this blade, an incision was made across a caudal vein of the tail. A total of 50µl of blood, 10µl at a time, was drawn up, using Costar Micropipettes. The blood was put into 1.5ml Eppendorf microtubes that contained 200µl of heparinised RPMI 1640 medium. The blood was therefor diluted 1 in 5 with heparinised RPMI 1640 medium. The heparinised RPMI 1640 medium was made up by drawing up 5ml of RPMI 1640 medium with a sterilised Promex syringe and needle into a 4.5ml Vacutainer (Becton Dickinson Vacutainer Systems, Sterile Interiors) that contained Lithium Heparin. This medium was made up under sterile conditions in a laminar flow cabinet. Cell counts were then performed on the blood cells to either determine the number of viable cells or to determine the volume of blood needed that would be incubated with the various metal salts.

2.3.4.2 Splenocyte Cultures

Female BALB/c mice were killed by cervical dislocation. The shrews were put into a dessicator (one at a time) containing water and chloroform to immobilise them. After the shrew showed signs of decreased movement, it was taken out of the dessicator and killed by cervical dislocation.

The spleen of the mice and shrews were then dissected out under aseptic conditions in a laminar flow cabinet. The spleen of the animals was removed aseptically with a pair of DuMont Tweezers, and placed into enough RPMI 1640 medium that contained 10% heat inactivated fetal calf serum to cover the bottom of a Petri dish. The fetal calf serum was heated at 65°C for 30 minutes to prevent complement reaction. The spleen was finely cut into small pieces using a pair of scissors and the spleen cell suspension was further diluted with 2.5ml of RPMI 1640 medium. The cell suspension was made up with the RPMI containing 10% heat inactivated fetal calf serum to contain $1.2x10^7 cells.ml^{-1}$.

Viability counts as described in section 2.3.5.2 with Trypan Blue in Phosphate Buffered Saline (PBS) were performed as well as an LDH assay using the spleen cells of the shrews that were exposed to 0 and 2µM of $CdCl_2$.

2.3.5 Cell counting and viability

A Neubauer Superior haemocytometer was used throughout the *in vitro* experiments and the Comet assay to determine the number of viable cells in the culture medium.

2.3.5.1 BALB/c

Two microlitres of blood and RPMI 1640 medium was added to 18µl of RPMI 1640 medium and 20µl of Trypan Blue in PBS in a 1.5ml Eppendorf microtube and mixed gently by tapping the tube slightly with a finger. The Trypan Blue in PBS was made up from a stock solution of 0.2% Trypan Blue. 400µl of this Trypan Blue was mixed with 600µl of PBS that contained no Ca^{2+} or Mg^{2+} ions. Approximately 10µl of the cell suspension and 10µl of the Trypan Blue was put onto each side of the haemocytometer. The number of viable white blood cells was counted in a total of six small blocks in the centre grid of the haemocytometer. The mean number of cells from the six small blocks on each side of the haemocytometer was taken and used to calculate the amount of cells present in 1ml of the cell suspension.

2.3.5.2 *Myosorex varius*

Ten microlitres of splenocyte cell suspension and 10µl of Trypan Blue in PBS was put onto each side of the haemocytometer. The number of viable splenocytes was calculated out of a total of 100 cells that were counted at each exposure (0µM or 2µM $CdCl_2$). Viable splenocytes colour green while dead cells colour blue.

2.3.6 Calculation of Whole Blood Cell Concentrations

The amount of whole blood cells present in 1ml of cell suspension is calculated as follows: $Mean\ number\ of\ cells * 25*10^4*20 = x$

Where x is the number of cells present in 1ml of cell suspension and $25*10^4$ is needed to calculate the number of cells per ml. 20 is the dilution factor (the blood was diluted 1 in 5 with heparinised RPMI 1640 medium).

From the amount of cells present in 1ml of cell suspension, the volume of cell suspension required for incubation purposes were calculated as follows.

For $5*10^6$ cells to be present in a well of the 96-well Nunclon multiplate, so that there would be a high enough density of cells to count after all the dilutions were done, the calculations are as follows: -

$$(x / 5*10^6) * 10^3 = y$$

Where y is the required volume of cell suspension in microlitres. To calculate the volume of cell suspension that would be enough to seed the desired number of wells, the following calculations were done: -

$$(y/40) * \{(\#\ of\ wells*40) + 20\} = z$$

Where z is the volume of cell suspension in microlitres. The volume of additional RPMI 1640 medium that is needed to make up the total volume of cell suspension, is subtracted from the final volume of cell suspension required $\{(\#wells*40) + 20\}$.

2.3.7 Treatment/Exposure of Whole Blood Cells *in vitro*

Forty microlitres of the finally diluted cell suspension was added to 10µl of metal salts or blank in each well of the 96-well multiplate. For each metal salt concentration, there were two replicates per mouse.

The blank wells contained 40µl of cell suspension and instead of 10µl of metal salts, the blank wells contained 10µl of heparinised RPMI 1640 medium. The seeded 96-well multiplate was then put into a plastic zippy bag and incubated in a Labcon growth chamber at 35°C - 37°C for two hours.

After two hours, the 96-well multiwell plate was removed from the Labcon growth chamber and zippy bag. The plate was gently tapped to resuspend the cells. 2µl of the cell suspension, 18µl of RPMI 1640 medium and 20µl of Trypan Blue in PBS were mixed in a 1.5ml microtube. Approximately 10µl of the cell suspension was put onto each side of the haemocytometer and cell counts were done again to determine the number of viable cells after the two-hour incubation period. The number of viable cells per ml was calculated again.

2.3.8 Statistical Analyses

Kolmogorov-Smirnov normality tests were done to determine whether or not the data were parametric.

One-way ANOVA's were done on all parametric data and either the Kruskal-Wallis one-way ANOVA on Ranks or the Mann-Whitney Rank Sum T-Test was performed on all non-parametric data. Dunn's method (all pairwise multiple comparison procedures) was performed as a post-hoc test. A P value of less than 0.05 was considered significant. The program Jandel Scientific on SigmaStat 2 was used for statistical analysis.

Chapter 3

The Transfer of Cd and Pb from Earthworms to Shrews

3.1 Introduction

Numerous studies have shown that heavy metals such as Cd and Pb are transferred from the environment via invertebrates to animals in higher trophic levels of the food chain (Johnson & Roberts 1978; Andrews, Johnson & Cooke 1984; Ma 1987; Dodds-Smith, Johnson & Thompson 1992). Heavy metals have been found to accumulate in earthworms (Ireland 1979; Ash & Lee 1980; Hartenstein, Neuhauser & Collier 1980; Hendriks, Ma, Brouns, de Ruiter-Dijkman & Gast 1995). In studies performed by Pankakoski *et al.* (1994b) on the shrew *Sorex araneus*, earthworms were found to be an important link between soil and secondary consumers (insectivores) in heavy metal transport.

According to Ma (1987) bioaccumulation studies provide information on pollutants in biotic systems that supplements the data obtained from the chemical analysis of abiotic samples. Choosing a suitable indicator species of environmental pollution in an ecosystem would depend on the trophic level that is to be investigated. Ma (1987) studied heavy metal bioavailability in a terrestrial decomposer subsystem, and chose the mole *Talpa europea* as an indicator species of metal bioavailability. Previous studies by Ma (1982) and (1983) were conducted at the first trophic level in a terrestrial decomposer subsystem, and he used lumbricid earthworms as indicator species.

Concern has been raised that the number of forest shrews, *Myosorex varius,* in the Stellenbosch region in the Western Cape in South Africa, might be dwindling as a result of increased urbanisation and varius anthropogenic activities (A. J. Reinecke *pers. com.*). The high pitched squeals of the forest shrew *Myosorex varius,* were a common sound heard in many gardens in Stellenbosch and the sight of a mother shrew with her little ones following her close behind, was often seen on many of the farm fields of the region in late Summer. This is not the case anymore.

Insectivores, like shrews, have an important ecological role to fulfil by keeping insect pest populations under control. Shrews are excellent indicator species of environmental contamination (Pankakoski, Koivisto, Hyvärinen & Terhivuo 1994a). They have a wide range of habitats, defend a small home range (Dippenaar 1997) and are easily collected. The high metabolic rate (MacDonald 1985; Churchfield 1990) and metal intake rates (Ma *et al.* 1991) of these small mammals, result in them being more exposed to xenobiotics than large mammals. Shrews are also carnivorous and therefor accumulate heavy metals in higher concentrations than herbivores (Churchfield 1990; Ma *et al.* 1991). They form an important part of the diet of some of the Canidae, Reptilia and Aves. The findings of this study (conducted in and around the Stellenbosch region) could indicate whether pollution levels of toxic heavy metals in our soils have reached levels that could effect not only shrews and other insectivores, but also animals higher up in the food chain - possibly also humans in the near future.

Ma (1987) quotes Moriarty (1983) as saying that the food chain involving earthworms and moles provides an example of a critical pathway for Cd and Pb. Studies done by various authors (Pankakoski *et al.* 1994b; Hendriks *et al.* 1995; Reinecke, Reinecke, Musilbuno & Chapman 2000) confirm this exposure route of heavy metals for other small mammals.

Ma (1987; 1989), Ma *et al.* (1991) and Pankakoski *et al.* (1994b) studied ground living small mammals that were exposed to Cd and Pb. They found that these small mammals were exposed to high levels of Cd and Pb in their natural environments. Reinecke *et al.* (2000) studied the transfer of Pb from earthworms to shrews in the Stellenbosch region in the Western Cape and found that the shrews were exposed to high levels of Pb in their natural environment.

It is significant to note that Pb appears to be the only toxic chemical pollutant to have accumulated in humans at mean concentrations that approach the threshold for potential clinical poisoning (Pain 1995). Lead accumulates mostly in the skeleton and brain of exposed organisms. The toxic effects of Pb have been shown to include blindness, kidney and liver damage, reduced brain weight and impaired motor skills (Demayo, Taylor, Taylor & Hodson 1982). The main clinical signs of Cd toxicity in animals are anaemia, retarded gonad development, enlarged joints, scaly skin, liver and kidney damage and reduced growth (Pain 1995).

Various anthropogenic activities like the use of pesticides and fertilisers on farmlands, and also the combustion of fossil fuels lead to the emission of Cd and Pb into the environment. Absorption of Pb through the skin is minimal. Following absorption, Pb moves into the bloodstream where it is taken up in soft tissues (primarily the kidneys and liver) within minutes, and accumulates eventually in bone tissue (Pain 1995).

Cadmium enters the environment via three routes: the refining and use of Cd, copper and nickel smelting and finally, via fuel combustion (Wren *et al.* 1995). It accumulates mostly in the kidney where it binds to the protein metallothionein as part of a detoxification mechanism (Andrews *et al.* 1984). According to Churchfield (1990) Cd becomes chemically transformed in its passage up the food chain and seems to be more readily absorbed through the gut walls of invertebrates and their predators than by plants.

The aims of this study were:

i. To compare the Cd and Pb contents of soils, earthworms, and shrews (*M. varius* and *C. flavescens)* in the Stellenbosch region in the Western Cape, South Africa;
ii. To study the transfer of Cd and Pb from the earthworms to the shrews, not only in the animals' natural environment, but also in the laboratory, from Cd and Pb-exposed cultures of earthworms kept there;
iii. Metal contents in the tissues of the bone, brain, gut, heart, kidneys, liver, lungs, muscles and skull of the shrews were determined as an indication of the distribution of these two metals in the bodies of these animals.

3.2 Material and methods

3.2.1 Animals

The control group of shrews consisted of one male and one female of the species *M. varius* and one female of the species *C. flavescens*. They were all caught in Raithby near a highway, and in a garden in a residential area in Stellenbosch. These shrews were fed control earthworms (*Eisenia fetida*) in the lab. The two *M. varius* shrews died suddenly after only three days. The Crocidura shrew, survived for 21 days.

The exposure group of the species *M. varius*, from the Raithby area, were fed either Cd or Pb contaminated earthworms. The Cd exposure group consisted of three females. One of the shrews was exposed in this way for a period of 21 days, while the remaining two were exposed to Cd for a period of two and 14 days respectively.

The Pb exposure group of shrews were exposed in the lab to Pb for a period of 14 days. This exposure group consisted of one male and one female shrew. The exposure periods for the two groups of shrews is not the same due to time constraints in which this study was conducted.

Food residues in the cages were removed daily and weighed. The cages were cleaned once a week and clean water was always available.

3.2.3 Samples for Metal Analyses

After the shrews had been exposed to the various metals for the above-mentioned periods of time, they were killed. One shrew at a time was put into a dessicator with chloroform until it was dead.

The following tissue samples were dissected from the dead shrews (both control and exposure shrews): femur, brain, gut, heart, liver, kidney, lung, muscle and skull. The tissue samples, whole invertebrates (Coleopterans and an Orthopteran) from the sites where the shrews were trapped; earthworms from the various cultures that were fed to the shrews while in the laboratory; as well as soil samples that were taken from the sites where the shrews were trapped were weighed in polytops on an electronic balance and frozen till needed for acid digestion for metal analysis.

3.2.4 Metal Analyses

The samples had to be acid digested to extract all metals from the samples and bring it into solution. To do this the tissue samples were defrosted and placed into test tubes. 10ml of 55% nitric acid was added to all the samples.

Soil samples were first dried for 48 hours in an oven at 70°C. The soil samples were then ground with a mortar and pestle and then sieved to obtain particles of uniform size. The soil samples were then centrifuged at 3000 rpm for five minutes. The supernatant was then decanted and acid digested in the same way that the tissue samples.

The samples were then left overnight in the test tubes. The following morning the samples were heated to 40-60°C for two hours and then again to 110-120°C for one hour. Perchloric acid (70%) (1ml for the tissue samples and 5ml for the soil samples) was also added after which the samples were left for two hours to cool down. After 5ml of distilled water had been added to the samples, the samples were heated at 100-120°C till white vapours were formed from the test tube. The samples were again left overnight. The samples were filtered that following day into 20ml volumetric flasks using Nr. 6 Whatman filterpaper and 0.45µm micropore filter paper. The samples were diluted with distilled water to the 20ml mark on the volumetric flasks. The samples were filtered for a second time with 0.45µm micropore filter paper into polypropylene containers and stored at 4°C until being analysed. Blank solutions were also made up to correct for the presence of any metals present in the distilled water.

Atomic absorption spectrophotometry (AAS) for Cd and Pb were done using a Varian-AA-1275 (Varian Techtron 1979) from the Department of Physics.

To calculate the concentration of the metals present in the samples after atomic absorption spectrophotometry, the following equation was used:

[{AAS sample reading (ppm)-AAS Blank reading (ppm)} x Volume (ml)]/mass (g)

Where the volume is 20ml and the mass is the mass of the animal material that was acid digested.

3.3 Results

Tables 3.1 and 3.2 show the Cd and Pb content of the soil and invertebrates that were taken from the sites where the shrews were trapped. From the tables it can be seen that there is both a higher Cd and Pb content in the soil (Figure 3.1) and earthworms (*Eisenia rosea*) from the farm in Raithby than in the Stellenbosch garden (Table 3.3).

Table 3.1 Cd content ($\mu g.g^{-1}$) in the soil (dry weight) and invertebrates (wet weight) found at the two sites where the shrews (*M. varius* and *C. flavescens*) were captured.

Control Stellenbosch	Cd	±SE	Control Raithby	Cd	±SE
Soil (n=3)	0.40	±0.35	**Soil (n=3)**	0.56	±0.14
***E. rosea* (n=6)**	n.d		***E. rosea* (n=7)**	1.88	±0.68
***Pheretima spp.* (n=3)**	0.37	±0.01	***Aporrectodea spp.* (n=4)**	0.55	±0.18
Coleoptera (n=4)	0.00	±0.00	**Orthoptera (n=1)**	0.12	

n.d Not Detected

Table 3.2 Pb content ($\mu g.g^{-1}$) in the soil (dry weight) and invertebrates (wet weight) found at the two sites where the shrews (*M. varius* and *C. flavescens*) were captured

Control Stellenbosch	Pb	±SE	Control Raithby	Pb	±SE
Soil (n=3)	0.15	±0.19	**Soil (n=3)**	25.85	±4.21
***E. rosea* (n=6)**	14.0	±8.57	***E. rosea* (n=7)**	77.19	±26.9
***Pheretima spp.* (n=3)**	21.4	±1.43	***Aporrectodea spp.* (n=4)**	25.4	±8.55
Coleoptera (n=4)	8.20	±1.30	**Orthoptera (n=1)**	6.24	

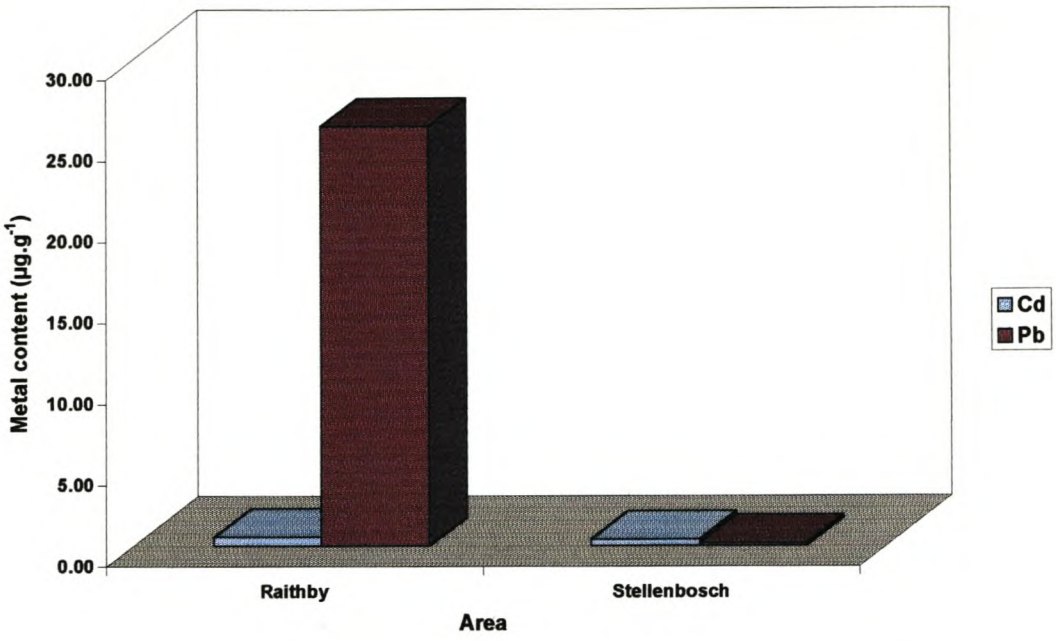

Figure 3.1 Cd and Pb content ($\mu g.g^{-1}$) (dry weight) in the soil sampled from Raithby and Stellenbosch where the shrews (*M. varius* and *C. flavescens)* were caught

Table 3.3 Metal content ($\mu g.g^{-1}$) (wet weight) in the food that was fed to the control shrews (*M. varius* and *C. flavescens*) and the laboratory exposed shrews (*M. varius*)

Food Item	Cd	Pb
Beef mince (n=1)	0.13	4.57
Ostrich mince (n=1)	0.24	5.93
Vitamin supplement (n=1)	0.12	4.14
***Control earthworms (n=1)**	1.22	27.5
***Contaminated earthworms (n=1)**	68.3	70.6

**E. fetida*

3.3.1 *Myosorex varius*

3.3.1.2 Cadmium

No Cd was found in the tissues that were analysed for Cd of the control shrews from Stellenbosch (Table 3.4). The control shrews from Raithby did however have Cd present in the tissues of the organs that were analysed. The highest concentration of Cd that was found in these shrews was in the skull. The female shrew having a higher Cd content in its skull ($26\mu g.g^{-1}$) than the male shrew ($21.1\mu g.g^{-1}$).

When compared to the control shrews from Raithby, the laboratory Cd-exposed group of shrews had lower concentrations of Cd present in their skulls. One of the Cd-exposed shrews, which was exposed in the laboratory to Cd for 14 days, had a Cd concentration of $127.0\mu g.g^{-1}$ in its skull. This was higher than any of the skull Cd contents of the control shrews (that were not exposed to Cd in the laboratory).

The shrew that was exposed in the laboratory to Cd for a period of 21 days had the highest concentration of cadmium in its kidneys. In a second shrew that was exposed in the laboratory to cadmium for 2 days, the muscle tissue had the highest Cd content.

The kidneys and liver of the Cd-exposed shrews had higher cadmium concentrations than either of the two control groups of shrews (Figure 3.2). The kidneys of the laboratory Cd-exposed shrews contained more cadmium than the liver. The Cd concentrations in all of the other remaining tissues that were taken from the laboratory Cd-exposed shrews were elevated in comparison to the basal levels that were present in both of the control groups. Only in the bone tissue of one of the shrews from the Cd-exposed group, was there a lower Cd concentration than either of the two control groups (and any of the two other Cd-exposed shrews). In this shrew, the muscle tissue accumulated the most Cd.

Table 3.4 Cd content (µg.g $^{-1}$) (wet weight) in the organs of the control and laboratory Cd-exposed shrews *M. varius*. The highest Cd concentration for each shrew is shown in bold in the appropriate column

Tissue	Female^{SC}	Male^{SC}	Female^{RC}	Male^{RC}	Female^{Cd}	Female^{Cd}	Female^{Cd}
*Days	0	0	0	0	21	14	2
#Bone	0.00	0.00	4.96	6.04	9.97	11.6	1.77
Brain	0.00	0.00	3.32	4.64	5.97	19.3	10.9
Gut	0.00	0.00	0.16	0.11	7.98	2.50	2.45
Heart	0.00	0.00	1.82	1.42	2.61	3.16	3.99
Kidney	0.00	0.00	2.37	1.90	**25.9**	14.7	4.56
Liver	0.00	0.00	0.38	0.54	9.72	3.39	0.97
Lung	0.00	0.00	1.21	2.90	4.71	6.21	5.28
Muscle	0.00	0.00	2.03	1.23	12.7	8.11	**30.3**
Skull	0.00	0.00	**26.0**	**21.1**	11.2	**127.0**	16.3

SC refers to the Stellenbosch control shrews (*M. varius*). RC refers to the Raithby control shrews (*M. varius*) and Cd to the Cd-exposed shrews (*M. varius*). #From the right femur. * Exposure times to Cd in the laboratory

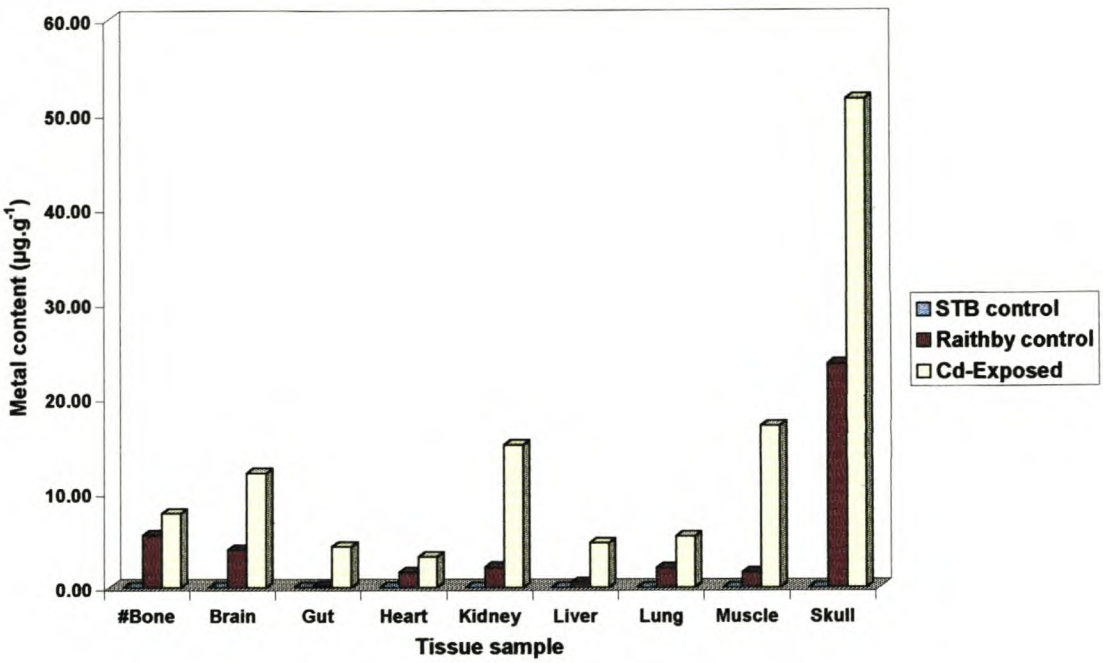

Figure 3.2 Pooled data of Cd content ($\mu g.g^{-1}$) (wet weight) in the tissue of the organs of the control shrews (*M. varius* and *C. flavescens)* from Raithby and Stellenbosch and from the laboratory Cd-exposed shrews. #From the right femur

3.3.1.3 Lead

The highest concentration of Pb in both of the control groups of shrews was found in the skull (Table 3.5). The Pb concentration of the bone was then second highest, followed by the brain Pb concentration.

The highest concentration of Pb in the laboratory Pb-exposed shrews was found in the bone (female shrew) and skull (male shrew). The second highest Pb concentration was found in the female laboratory Pb-exposed shrew's skull, and in the brain of the male laboratory Pb-exposed shrew.

The lowest concentration of Pb in both of the control groups of shrews and in the laboratory Pb-exposed group of shrews was found in the gut (Figure 3.3). Only in one shrew did this differ, viz. in the female control shrew from Stellenbosch where the lowest Pb concentration was found in the liver.

Table 3.5 Pb content ($\mu g.g^{-1}$) (wet weight) in the organs of the control and laboratory Pb-exposed shrews *M. varius*. The highest Pb concentration for each shrew is shown in bold in the appropriate column

Tissue	Female^{SC}	Male^{SC}	Female^{RC}	Male^{RC}	Female^{Pb}	Male^{Pb}
*Days	0	0	0	0	14	14
#Bone	----	18.6	238.0	302.0	**1292.7**	304.0
Brain	48.2	9.50	159.2	213.5	176.9	314.5
Gut	10.3	1.90	7.92	6.01	5.91	0.00
Heart	----	8.50	82.0	65.4	66.1	66.5
Kidney	29.1	11.2	109.0	98.5	80.7	110.7
Liver	7.10	1.30	17.5	24.4	15.0	9.16
Lung	20.8	6.80	59.2	127.5	123.2	10.7
Muscle	----	7.60	105.8	60.2	144.0	289.0
Skull	**80.0**	**77.2**	**1299.0**	**1031.6**	938.0	**2410.0**

SC refers to the Stellenbosch control shrews (*M. varius*). RC refers to the Raithby control shrews (*M. varius*) and Pb to the lead-exposed shrews (*M. varius*). The dashed lines (----) represent values that are not available. #From the right femur. * Exposure times to Pb in the laboratory

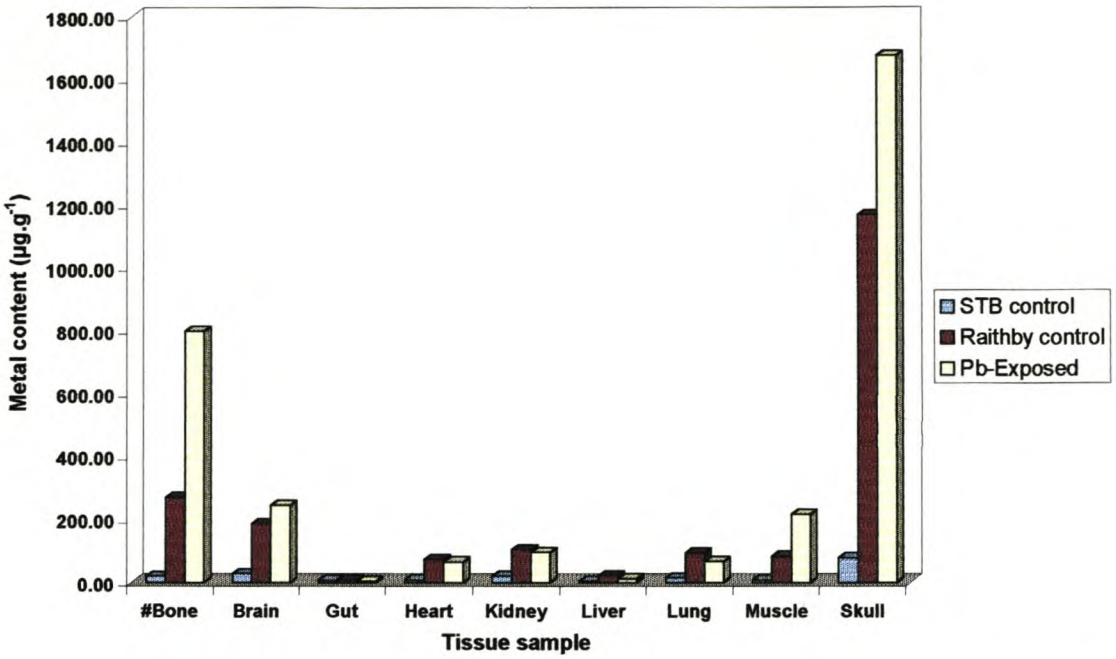

Figure3.3 Pooled data of Pb content ($\mu g.g^{-1}$) (wet weight) in the tissue of the organs of the control shrews (*M. varius* and *C. flavescens)* from Raithby and Stellenbosch and from the laboratory Pb-exposed shrews. #From the right femur

3.3.2 Crocidura flavescens

3.3.2.1 Cadmium

These results are based on only one individual. The highest concentration of Cd was found in the skull (Table 3.6). In all of these tissues that were analysed for Cd from this shrew, the cadmium levels were elevated when compared to that of either of the control groups of shrews (*M. varius*). The kidneys of the Crocidura shrew had higher levels of Cd than the liver (Figure 3.4). The pattern of Cd accumulation in the shrew is skull>brain>bone>kidney>liver, lung and gut.

3.3.2.2 Lead

The highest concentration of Pb was found in the skull (Table 3.7). The brain, muscle and bone tissues followed this. When compared to the female control shrew (*M. varius*) from Raithby, the Pb concentration in the skull, brain and muscle tissue of the exposed shrew was

higher than the basal levels found in the female control shrew. These values were however lower than those of the male control shrew (except in the muscle tissue) (Figure 3.5).

Table 3.6 Cd content ($\mu g.g^{-1}$) (wet weight) in the organs of the control shrews *M. varius* and *C. flavescens*. The highest Cd concentration for each shrew is shown in bold in the appropriate column

Tissue	Female^{SC}	Male^{SC}	Female^{RC}	Male^{RC}	Female^{C}
*Days	0	0	0	0	0
#Bone	0.00	0.00	4.96	6.04	9.30
Brain	0.00	0.00	3.32	4.64	11.4
Gut	0.00	0.00	0.16	0.11	0.32
Heart	0.00	0.00	1.82	1.42	3.55
Kidney	0.00	0.00	2.37	1.90	8.75
Liver	0.00	0.00	0.38	0.54	1.79
Lung	0.00	0.00	1.21	2.90	1.77
Muscle	0.00	0.00	2.03	1.23	5.38
Skull	0.00	0.00	**26.0**	**21.1**	**47.1**

SC refers to the Stellenbosch control shrews (*M. varius*). RC refers to the control shrews (*M. varius*) from Raithby and C to the control shrew *C. flavescens*. #From the right femur. * Exposure times to Cd in the laboratory

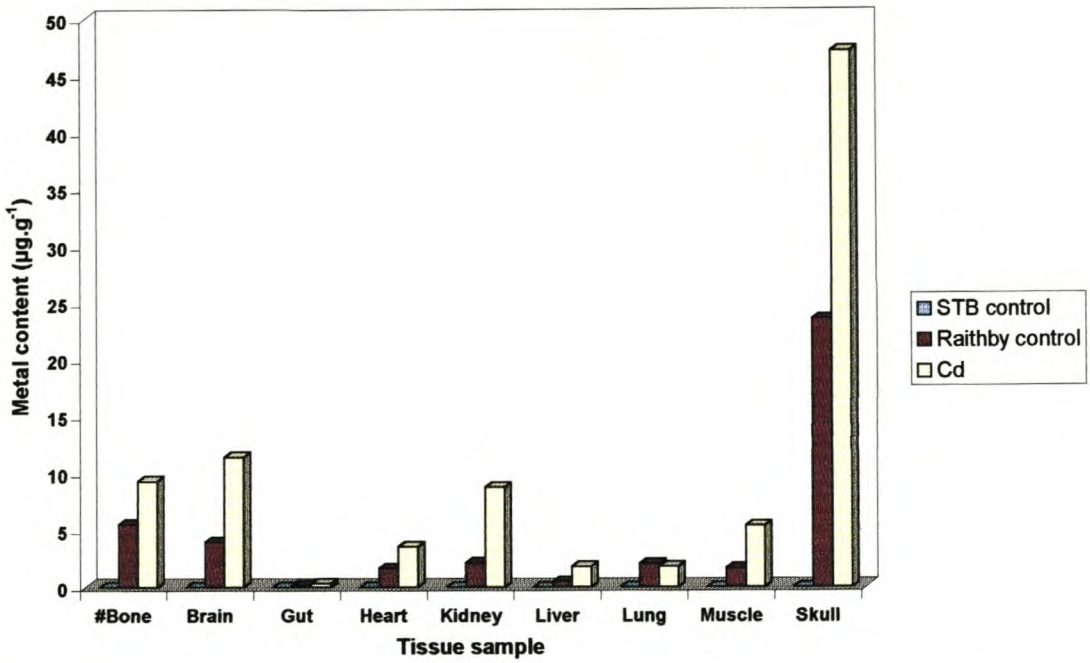

Figure 3.4 Pooled data of Cd content ($\mu g.g^{-1}$) (wet weight) in the tissue of the organs of the control shrews (*M. varius)* from Raithby and Stellenbosch and from the control shrew *C. flavescens.* #From the right femur

Table 3.7 Pb content ($\mu g.g^{-1}$) (wet weight) in the organs of the control shrews *M. varius* and *C. flavescens*. The highest Pb concentration for each shrew is shown in bold in the appropriate column

Tissue	Female^{SC}	Male^{SC}	Female^{RC}	Male^{RC}	Female^{C}
*Days	0	0	0	0	0
#Bone	----	18.6	238.0	302.0	141.9
Brain	48.2	9.50	159.2	213.5	185.6
Gut	10.3	1.90	7.92	6.01	7.21
Heart	----	8.50	82.0	65.4	57.9
Kidney	29.1	11.2	109.0	98.5	54.7
Liver	7.10	1.30	17.5	24.4	16.1
Lung	20.8	6.80	59.2	127.5	32.5
Muscle	----	7.6	105.8	60.2	170.0
Skull	**80.0**	**77.2**	**1299.0**	**1031.6**	**776.0**

SC refers to the Stellenbosch control shrews (*M. varius*). RC refers to the control shrews (*M. varius*) from Raithby and C to the control shrew *C. flavescens*. The dashed lines (----) represent missing values. #From the right femur. * Exposure times to Pb in the laboratory

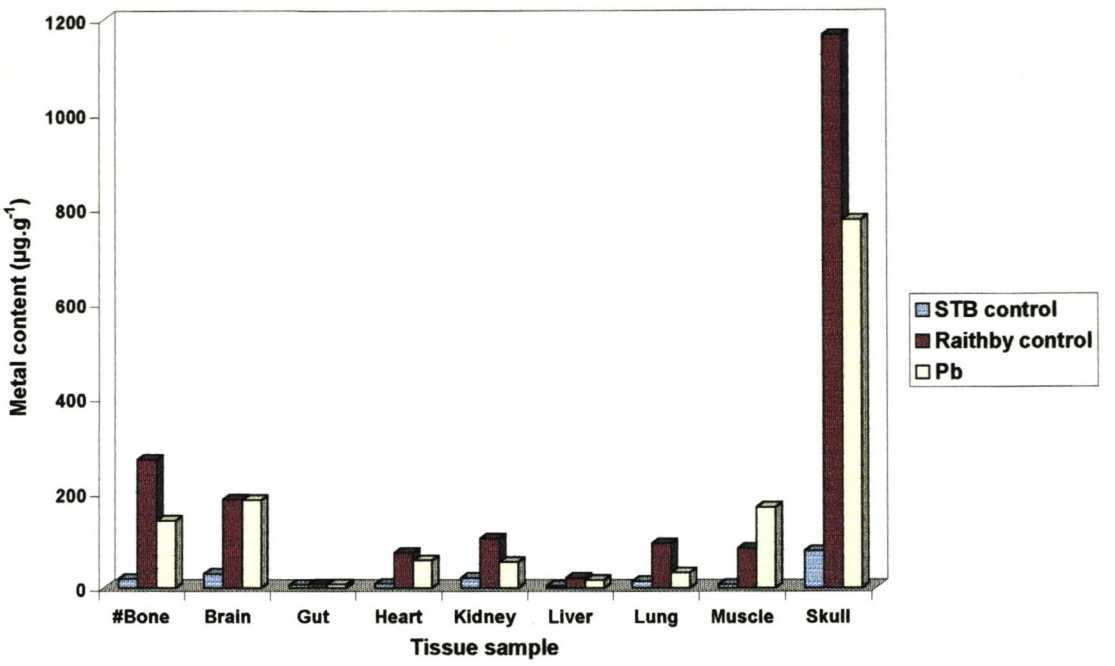

Figure 3.5 Pooled data of Pb content ($\mu g.g^{-1}$) (wet weight) in the tissue of the organs of the control shrews (*M. varius)* from Raithby and Stellenbosch and from the control shrew *C. flavescens.* #From the right femur

3.4 Discussion

Raithby is a small farming community situated 16km outside the town of Stellenbosch near to a busy urban highway. The residents farm mainly with vegetables for their own consumption on their smallholdings. The high Cd (Table 3.1) and Pb (Table 3.2) concentrations found in the soil and invertebrates that were sampled in this area compared to the levels of Cd and Pb found in the soil and invertebrates samples from the immediate Stellenbosch area, may be attributed not only to the location where the shrews were caught, but also to the possible injudicious use of pesticides and fertilisers on the farms in Raithby. The proximity of this small subsistence farming community to a chemical producing plant should also be noted, although its significance was not pursued during the present study.

Four years prior to the present study, a chemical fire broke near Raithby. It is quite possible that the high levels of Pb found in the soil from Raithby might be attributed to the deposition of atmospheric Pb that occurred as a result of that fire where vast amounts of sulphates were also released into the air. Lead found in the soil, as a result of the deposition of atmospheric Pb, is relatively immobile compared to Pb in air and water. Soils have also been described as "sinks" for anthropogenically emitted Pb (Pain 1995). This might explain the high levels of Pb found in the soil in Raithby (Figure 3.1).

No pesticides or fertilisers had been used for many years in the garden where the Stellenbosch shrews were trapped. The soil from both sites (Raithby and Stellenbosch) where these shrews were caught is relatively clean by European and United Nations permissible limits for Cd and Pb in soil, revised by Sittig (1994).

Cadmium and Pb are by-products of many industrial processes (Wren *et al.* 1995). The shrews from the garden in the residential area in Stellenbosch would have been exposed to very little Cd and Pb from industrial sources in their environment, in comparison to the Raithby shrews. The emission of Cd and Pb as a result of industrial activity in/near Raithby would also be minimal.

Pesticides run off into the soil and manure is worked into the soil by agricultural activities (e.g. tillage, crop rotation etc.). Atmospheric Cd and Pb released into the environment, e.g. by the combustion of fossil fuels, settles into the soil where it is taken up by invertebrates. Shrews are frequently found in areas associated with agricultural activities by nature of their habitat and ubiquity.

No Cd was found in the tissues of the field shrews from Stellenbosch (Tables 3.4 and 3.6). There was however, Cd in the tissues of the Raithby field shrews (Tables 3.4 and 3.6). The high levels of Pb in the kidneys of the control shrews from the field (Tables 3.5 and 3.7) show that these animals are exposed to high levels of Pb in their natural environments. This was especially evident in the shrews that were caught in Raithby (Figures 3.3 and 3.5). The tissues of the Crocidura shrew, fed control earthworms in the laboratory, from Raithby had higher levels of Cd than the control shrews (*M. varius)* from either Stellenbosch or Raithby (Figure 3.4). The levels of Pb in the kidneys and liver of the Crocidura shrew compared to the levels of Pb in the kidneys and liver of the Raithby control shrews were lower (Figure 3.5).

By preying on invertebrates, shrews incorporate heavy metals in their bodies (Churchfield 1990). Earthworms are a major part of the diet of shrews (Churchfield 1990; Ma *et al.* 1991; Dickman 1995). Various authors have shown that these invertebrates, responsible for maintaining important physical soil characteristics and processes, are capable of accumulating heavy metals (Ireland 1979; Ash & Lee 1980; Hartenstein *et al.* 1980; Hendriks *et al.* 1995).

Ma *et al.* (1991) reported high metal intake rates of Cd (3-16$\mu g.g^{-1}$) and Pb (19-53$\mu g.g^{-1}$) per day in shrews that were exposed to Cd and Pb in terrestrial environments. This was largely due to the consumption of contaminated earthworms. The control earthworms used during the present study that were fed only cattle manure also had Cd and Pb in their bodies (Table 3.3). Cattle are herbivorous and so the presence of these metals in the manure is probably as a result of metal accumulation from plants. This is an indication that the soil in the pastures (also in the Stellenbosch area) where the cattle were feeding is also contaminated with Cd and Pb.

Ma 1989 and Ma *et al.* (1991) reports that Cd levels of 120$\mu g.g^{-1}$ and Pb levels of 25$\mu g.g^{-1}$ are critical for the kidneys of small mammals and will result in adverse health effects. In the present study, the highest levels of Cd and Pb that were found in the control shrews were 2.37$\mu g.g^{-1}$ (Table 3.4) and 109.0$\mu g.g^{-1}$ (Table 3.5) of Cd and Pb respectively. The highest levels of Cd and Pb found in the kidneys of the Cd and Pb-exposed shrews in the laboratory were 25.9$\mu g.g^{-1}$ (Table 3.4) and 110.7$\mu g.g^{-1}$ (Table 3.5) Cd and Pb respectively. Ma 1989 and Ma *et al.* (1991) only report on Cd and Pb levels from animals previously exposed to the metals in their natural environments and not from laboratory exposed animals. The principal target organs in which Cd accumulates have been identified from previous

studies as being the kidneys and liver (Johnson *et al.* 1978; Andrews *et al.* 1984). The Cd-metallothionein complex is stored mainly in the kidneys and liver and acts as a detoxification mechanism by removing Cd from the general circulation in the body (Andrews *et al.* 1984). In the present study, in only one shrew did the kidney accumulate the most Cd in comparison to the other organs that were analysed (Table 3.4).

While studying the shrew *S. araneus* Andrews *et al.* (1984) found that when dietary Cd levels were elevated, the kidney's status as primary target organ for Cd accumulation changed. The liver accumulated more Cd than the kidneys. He proposed that this was a consequence of kidney damage and that the liver takes over the function of Cd storage, the kidney being particularly affected by Cd. In the present study, the kidneys of the shrews that were exposed to Cd in the laboratory, as well as the Crocidura shrew that was fed control earthworms in the laboratory, accumulated more Cd than the livers of these shrews (Figures 3.2 and 3.4). The laboratory Cd-exposed earthworms had higher levels of Cd in their bodies than the earthworms found at the sites in Raithby where these shrews were caught (Tables 3.1 and 3.3).

Johnson *et al.* (1978) found that Pb accumulates in a well-established pattern viz. bone>kidney>liver>brain and muscle. In the present study, elevated levels of Pb were observed in the skull, bone, brain and muscle tissue of the laboratory Pb-exposed shrews. The pattern of Pb accumulation was therefor found to be skull/bone>brain>lung>kidney>liver which indicate that the highest levels of Pb are due to long-term exposure and the lower levels of Pb due to more recent short-term exposure of the shrews to Pb.

Lead is stored in the bone in a metabolically inactive form. Johnson *et al.* (1978) found that in small mammals with long-term exposure to Pb, the highest Pb concentrations were in the bone tissue. In the present study, the field control shrews from Stellenbosch and Raithby, as well as the laboratory control shrew from Raithby, had the highest concentration of Pb in their skulls, followed by the bone tissue (Tables 3.5 and 3.7). The elevated levels in the bone tissue of the female shrew and in the skull of the male shrew that were exposed to Pb in the laboratory show a high, long-term exposure to Pb. In the present study, no differences regarding accumulation of Pb (or Cd) between the sexes were observed. Evidence for sex differences with regard to the accumulation of Pb between the sexes were not observed in a study on the shrew *S. araneus* performed by Pankakoski *et al.* (1994b).

According to Ma (1996) Pb absorped after inhalation or ingestion, moves in the blood stream to where it is taken up in the kidneys and liver within minutes. Pain (1995) stated that Pb in soft tissues indicates relatively recent exposure. In the present study the accumulation pattern of Pb typically indicated short-term exposure, i.e. brain, muscle, kidneys and liver (in that order) in the Pb-exposed shrews in the laboratory.

Lead interferes with the central nervous system after its passage through the blood stream (Ma 1996). Symptoms of long-term Pb exposure in mammals include hyperactivity, impaired motor co-ordination and abnormal social behaviour (Pain 1995). In a preliminary study on the transfer of Pb from earthworms to shrews, done in the laboratory, it was found that an individual shrew (*C. flavescens*) lost the ability to co-ordinate its motor skills. This was after 100 days in the laboratory and 12 days of actual exposure to live Pb-contaminated earthworms (*E. fetida)*. The concentration of Pb in the brain of this shrew was 557$\mu g.g^{-1}$. In the present study, the brain tissue is only the second highest accumulator of Pb (after the skull and bone tissue) (Table 3.5). In this study, the highest concentration of Pb was 314.5$\mu g.g^{-1}$ (Table 3.5) in the brain of the laboratory Pb-exposed shrew. The highest concentration of Pb in the control shrews from Raithby that were not exposed to Pb in the laboratory was 213.5$\mu g.g^{-1}$ (Table 3.5). After 14 days of continuous exposure to Pb contaminated earthworms, the levels of Pb in the brain seem to be higher than the basal levels of Pb found in the brains of the control shrews. Perhaps this is because before the shrews that were exposed to Pb in the laboratory were killed, the Pb is still circulating in the blood stream and passing through the blood-brain barrier.

Chapter 4

The Cytotoxic Effects of Cd, Pb and Zn in Mouse and Shrew Cells *in vitro*

4.1 Introduction

According to Balls and Clothier (1992), cytotoxicology is the study of toxic effects of chemicals and other materials at the cellular level. These authors define cytotoxicity as being the expression of adverse effects on the structure and/or functional properties of one or more cell components. General cytotoxicity deals with the result of toxic effects on structure and function of components that is common to all cells of the body e.g. DNA molecules, chromosomes, mitochondria, cytoskeleton and various membranes. Caldwell (1993) defines cytotoxicity as cell death. He states that it is the consequence of exposure to a harmful chemical.

Cadmium is an ubiquitous toxic heavy metal (Kostial 1986). Sources of environmental Cd include the combustion of fossil fuels, manufacture of paints and batteries and the smelting and refining of Zn and Pb ores (Pain 1995; Wren *et al.* 1995). Cadmium is a non-essential element in animals and the detection of this metal in the body fluids or tissues of animals suggests environmental exposure (Chowdhury & Chandra 1987). According to Jones & Cherian (1990), Cd enters the body following inhalation of airborne Cd or ingestion of contaminated foods. Once in the bloodstream it is deposited mainly in the liver and kidneys.

The major sources of environmental Pb contamination results from commercial uses of Pb containing compounds like plastics, putty, paint, batteries and ceramic glazes (Haley 1968). Like Cd, the burning of fossil fuels and the airborne effluent of Zinc and Nickel smelters also contributes to environmental Pb contamination (Haley 1968). Following inhalation of airborne Pb and ingestion from contaminated foods, it is taken up in the bloodstream before finally being deposited in the bone tissue. These two heavy metals interfere with the actions of mixed-function oxidases and phophorylases and affect the transport of ions across cell and mitochondrial membranes (Segner & Braunbeck 1998).

It is known that heavy metal toxicity have effects at the cellular level (De Ruiter and Mailänder 1985; Steffensen, Mesna, Andruchow, Namork, Hylland & Anderson 1994; Koropatnick & Zalups 1997). According to Ratcliffe, McElhatton & Sullivan (1993), Cd and Pb are cytotoxic.

Zinc is a trace element (Ganong 1995b) and is required for normal cellular development and functioning in concentrations that is not toxic to the cells (Koropatnick & Zalups 1997). It has also been shown that Zn stabilises plasma and lysosomal membranes (Bettger & O'Dell 1981). Animal studies have shown that Zn can at least partially prevent some known toxic effects of Cd (Webb 1972; Nath *et al.* 1984; Harris 1991; Endo *et al.* 1997).

Human exposure to environmental pollutants is often exceedingly complex (Shugart *et al.* 1989a) and therefor small mammals have frequently been used to monitor the presence and bioavailabilty of metals in the environment. By choosing the shrew, which is a common carnivorous mammal on the farm-fields in Stellenbosch, one has a reliable bioindicator of pollution in a terrestrial ecosystem.

Van Gestel & Van Brummelen (1996) defined a bioindicator as an organism that gives information on the environmental condition of its habitat by its presence, absence or behaviour. Pankakoski *et al.* (1994a), while studying the suitability of shrews as appropriate bioindicators of heavy metal pollution in southern Finland stated that indicator species should occupy a wide range of habitats and be abundant enough to obtain an adequate sample size. Reinecke *et al.* (2000) found that shrews from the Stellenbosch area were exposed to exceptionally high levels of environmental Pb and Pankakoski *et al.* (1994a) found that the common European shrew, *S. araneus*, was a reliable bioindicator for heavy metal pollution. In this study, the *in vitro* effects of Cd, Pb and Zn on mouse were studied. Not enough shrews could be caught to complete *in vitro* studies on shrew cells, and so a recognised strain of laboratory mice were used to complete the *in vitro* studies. Shrew cell cultures were only used for the LDH assay, using splenocyte cultures.

Butler (1996) defines cell culture as the process whereby cells that were removed from animal tissue or whole animals, are grown and supplemented with nutrients and growth factors. This process takes place outside of the intact animal (*in vivo* or in life), usually in some sort of culture container (*in vitro* or in glass).

The advantages of *in vitro* systems are that test cells or organisms are isolated from homeostatic and hormonal control (Gad 1993). The individual components of a system can be examined separately and in detail (Peakall 1992), and according to Butler (1996), one is able to investigate the normal physiology and biochemistry of cells, and one is also able to test the effects of compounds on specific cell types. Cell cultures contain cells of only one type, but the cells may or may not be genetically identical. Accurate dosing of the test substance is also possible and the results are quantifiable (Gad 1993). Experiments using cell cultures also greatly reduce the number of animals needed for animal experiments (Freshney 1992; Gad 1993). The cells in these cultures act as independent units, much as fungi do. The cells are capable of division by mitosis and cell population growth continues until some factor limits it e.g. nutrient depletion.

In toxicity testing, cell culture techniques allow for a greater understanding of the effects of a particular compound on a specific cell type e.g. hepatocytes. Routine toxicity testing is less expensive in culture than in the live animal (Butler 1996). Primary cell culture have been used in this study because of the loss of organ-specific functions that have been observed when established cell-lines have been used (Freshney 1992). Primary cell cultures contain cells that are taken directly from an animal into culture while an established cell line is a cell culture that consists of cells of many generations (Butler 1996).

According to Vallee & Ulmer (1972) and Stacey & Klaassen (1980), cell membrane induced damage by heavy metals is an indication of the cytotoxic effect of heavy metals. The loss of viability of cells is indicated by a damaged cell membrane. Cell viability measures the proportion of live cells in culture and gives an indication of the cells' ability to divide or to perform normal cell metabolism.

In a study by Steffensen *et al.* (1994) on the cytotoxic effects of heavy metals on human peripheral immune cells, trypan blue exclusion was chosen as the endpoint for cytotoxicity following Müller (1984). Müller (1984) stated that trypan blue exclusion is an easily performed test for plasma membrane integrity and therefor of cell viability. Membrane integrity, being the most common measurement of cell viability at the time of assay gives an estimate of instantaneous damage or progressive damage over a few hours (Wilson 1992). The viability is used to measure the degree of loss of membrane integrity and is based on the principle that positive staining with trypan blue is synonymous with a loss of membrane integrity and equitable with eventual cell death (Borgs, Dennis, Way, Witte, Case, Ramirez & Witte 1992). Trypan blue is added to the cell suspension before counting. The dye penetrates the membrane of non-viable cells and stains the non-viable cells blue (Butler 1996).

The lactate dehydrogenase (LDH) assay that was performed in this study for measuring membrane integrity was used to confirm the results obtained from the dye exclusion procedure using trypan blue. LDH leaks from damaged cell membranes of non-viable cells (Butler 1996). To measure the viability of cells, the LDH content in the culture medium is measured was as a proportion of the total LDH content of the culture.

The effects of the heavy metals on DNA synthesis were also investigated. Chemically induced damage to the nucleic acids of mammalian cells generally result in unscheduled DNA synthesis by the cell's enzymatic systems to repair the damage (Dinsdale & Williams 1977). The incorporation of tritiated ([^{3}H]) Thymidine into newly synthesised DNA was measured as an extra parameter of cell viability. Measuring the rate of DNA synthesis is dependent on the rate of incorporation of [^{3}H] Thymidine. The incorporation of this amino acid into DNA has been used successfully as an index for cytotoxicity.

The aims of this study were therefore to determine the effects of the heavy metals Cd and Pb, and the trace element Zn on the cell viability of mouse and shrew cells. The responses to metals regarding their effects on a cells' viability and the rate of DNA synthesis can be used as biomarkers of pollution induced stress that possibly have not yet effected irreversible adverse effects in animals exposed to heavy metals.

4.2 Materials and Methods

4.2.1 Animals

Twelve female BALB/c mice were used in this study, as well as a female shrew of the species *Myosorex varius.*

4.2.2 Cell Preparation

Blood was obtained from the caudal vein of the mice and cultured as described in sections 2.3.4.1. and 2.3.4.2. Cells for the mouse and shrew splenocyte cultures were obtained as described in section 2.3.4.2. and in section 2.3.4.7.

4.2.3 Cell Culture Conditions

A cell density of $5x10^6$ cells/ml was used throughout this study for the whole blood cultures of the mice. $1.2x10^6$ cells/ml was used in the splenocyte cultures of the mice and shrews. Splenocyte cultures from both the mice and shrews were used for the LDH assays. For the whole blood cultures, 40µl of this volume of cells were pippetted with Costar Micropippettes into each well of a 96-well Nunclon multiplate.

4.2.4 Metal Solutions

Metal ion ($CdCl_2$, $PbCl_2$ and $ZnCl_2$) stock solutions were made up in distilled water to a concentration of 10mM. This metal ion stock solution was then further diluted with RPMI 1640 medium before addition to the cell suspensions to obtain the desired exposure concentrations of the metals. The Cd, Pb and Zn solutions were added to each well of the 96-well multiplate containing 40µl of cell suspension. 10µl RPMI 1640 medium was added to the blank wells. The negative controls were the cells that were exposed to RPMI 1640 medium instead of to the metals.

4.2.5 Range Finding Test

The effects of $CdCl_2$ and $PbCl_2$ on the cell viability of mouse whole blood cells were investigated by exposing the cells to 20µM of $CdCl_2$ and $PbCl_2$ for two hours at 37°C.

A Zn dilution curve was constructed as well. Cells were exposed to $ZnCl_2$ concentrations in the range of 0µM, 2.5µm, 5µM, 10µM and 20µM and then incubated for two hours at 37°C in a Labcon growth chamber. Viability tests with 0.2% trypan blue in PBS

were then performed as described in section 2.3.5 to find out at which concentration(s) Zn had an optimally stabilising effect on the cell membranes of the mouse whole blood cells.

4.2.6 Cell viability by LDH

After the mouse and shrew splenocyte cultures were exposed to the metals salts for a two hour period at 37°C, the cell culture supernatants were collected.

10µl of the supernatant was added to 100µl of Pyruvate substrate, Catalog No. 500L-1, containing 1 mg/ml NADH (from the Mannheim-Boehringer LDH kit). The mixture was then incubated for 30 minutes at 37°C in a microplate incubator (Solo Denley Microplate Incubator). After 30 minutes, a standard curve was constructed using various volumes of distilled water and Pyruvate substrate, as described in the LDH Mannheim-Boehringer kit (Table 4.1). 100µl of the Sigma colour reagent, Catalog No. 505-2 from the kit was then added to all the wells containing the cell samples and substrate and the standard curve dilutions.

Standard curves were constructed for both the mouse and shrew data. After 20 minutes at room temperature, the optical densities of the wells were read using a multiskan plate reader (Labsystems Multiskan MS Plate Reader). The values of the unknown samples were read off the standard curve. See figure 4.1 for a diagrammatic representation of the LDH assay.

Table 4.1 Table of the volumes of Pyruvate substrate, Catalog No. 500L-1, containing 1 mg/ml NADH, and distilled water that were used to construct the standard curves for mouse and shrew data

Tube No.	Pyruvate Substrate Catalog No. 500L-1 (µl)	Water (µL)	LDH Activity (B-B Units/mL)
1	100	10	0
2	80	30	280
3	60	50	640
4	40	70	1 040
5	20	90	1 530
6	10	100	2 000

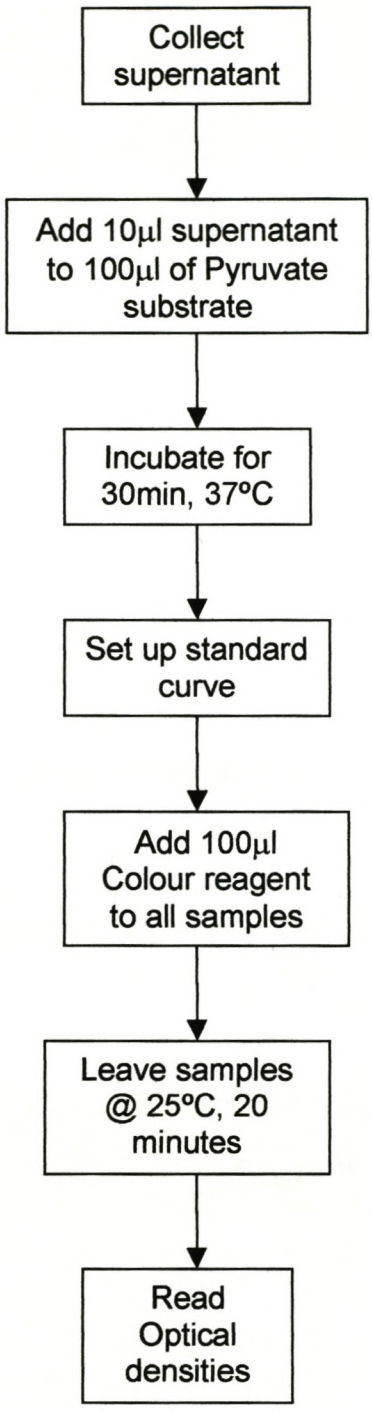

Figure 4.1 Flow Diagram for LDH assay as described in the Mannheim Boehringer kit

4.2.6 Cell viability by Trypan Blue exclusion

Cell viability was determined for the cells of the cultures that were exposed to the metal salts for two hours at 37°C in a Labcon growth chamber as described in section 2.3.5. The cells were counted in a Neubauer Superior haemocytometer.

4.2.7 DNA synthesis

Mouse whole blood cell cultures were exposed (refer to section 2.3.4.1) to 20µM of Cd, Pb and $ZnCl_2$ *in vitro*, while the shrew splenocyte culture was exposed, *in vitro,* to 2µM of Cd and $PbCl_2$ (refer to section 2.3.4.2).

To determine the rate of DNA synthesis, 2.5µl of [^{3}H] Thymidine was added to the cell cultures before incubation for two hours at 37°C in a Labcon growth chamber.

At the end of the incubation period, the mouse whole blood and shrew splenocyte cell suspensions were transferred to microfuge tubes from the 96-well multiplates. The wells were rinsed with 200µl of distilled water that was then added to the respective microfuge tubes. 250µl of 20% Trichloroacetic acid (TCA) was added to each tube. This suspension was mixed in a Vortex whirlimix and centrifuged (UniEquip Centrifuge UEC3) at 200g for 10 minutes.

The supernatants were removed and 200µl of distilled water and 125µl of 20% TCA was added to the cell pellet and mixed. The cell pellets were resuspended by recentrifugation (200g for 10 minutes). The supernatants were removed and the step was repeated once more. After the third centrifugation step (200g for 10 minutes), the supernatants were removed and 250µl of 1M NaOH was added to the cell pellet to hydrolyse it.

After an overnight incubation at room temperature in a fume cupboard, the hydrolates were resuspended in the NaOH already present in the microfuge tubes and 25µl of concentrated HCl was added to neutralise the NaOH. The cell suspensions were transferred to counting tubes and 4ml of scintillating fluid were added to each tube.

The amount of [^{3}H] Thymidine that was incorporated into newly synthesised DNA was then counted by a Varian Scintillating β-counter. See figure 4.2 for a diagrammatic representation of the DNA synthesis assay.

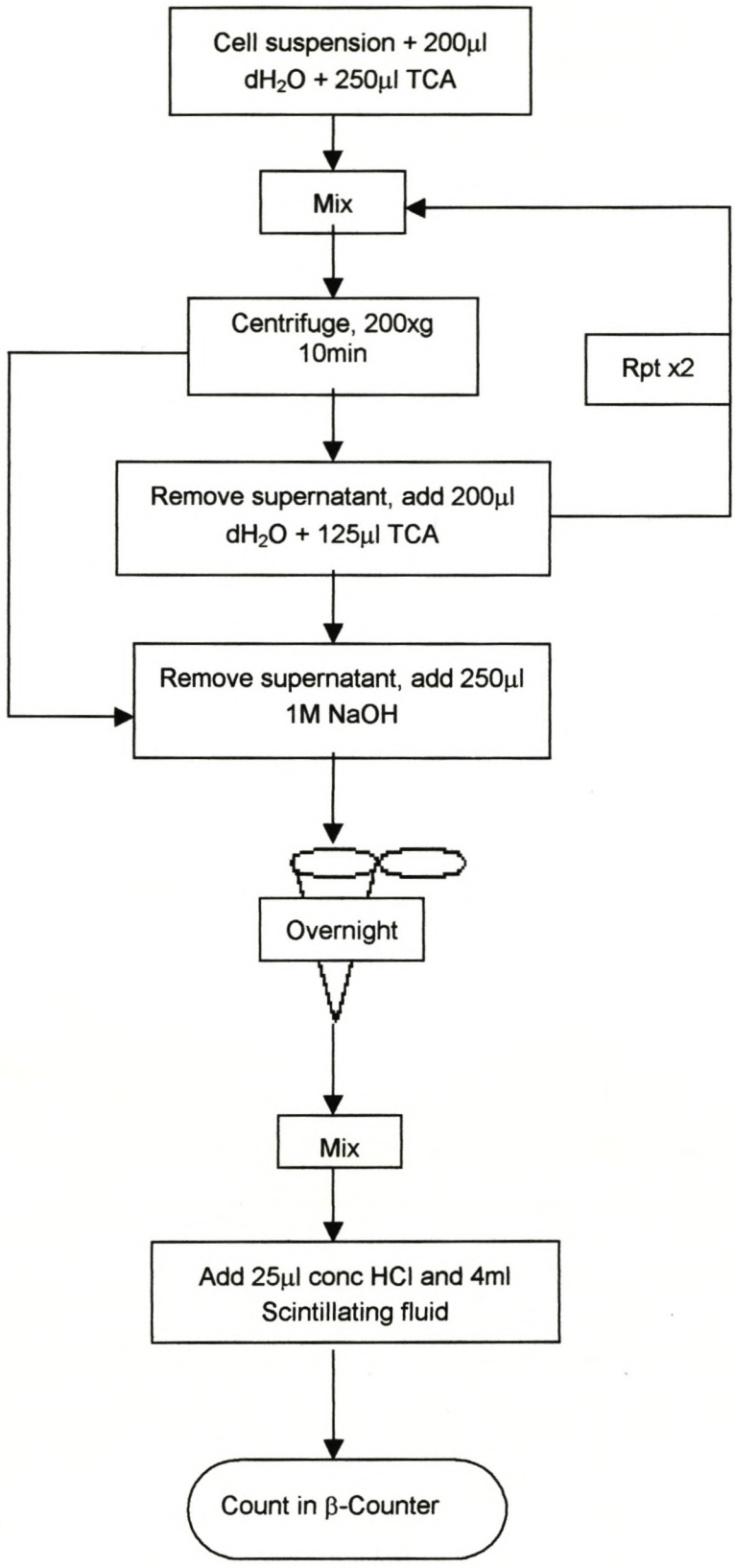

Figure 4.2 Flow Diagram of the DNA synthesis protocol after incubation of cells with [^{3}H] Thymidine

4.3 Results

4.3.1 Viability tests with LDH

Results summarised in Table 4.2 show that the exposure of mouse splenocytes to 20µM of $CdCl_2$, $PbCl_2$ and $ZnCl_2$ did not induce LDH secretion into the culture medium after the two hour incubation period (Figure 4.3).

Table 4.2 Optical densities measured at 405nm for the LDH assays of mouse splenocytes from mouse splenocyte cultures incubated at 37°C and treated with 0 or 20µM of $CdCl_2$, $PbCl_2$ or $ZnCl_2$

Individual #	LDH Control	LDH Cd	LDH Pb	LDH Zn
1	2.037	2.111	ND	ND
2	2.172	2.082	2.109	2.342
3	2.343	2.577	2.534	2.107

ND- Not Determined

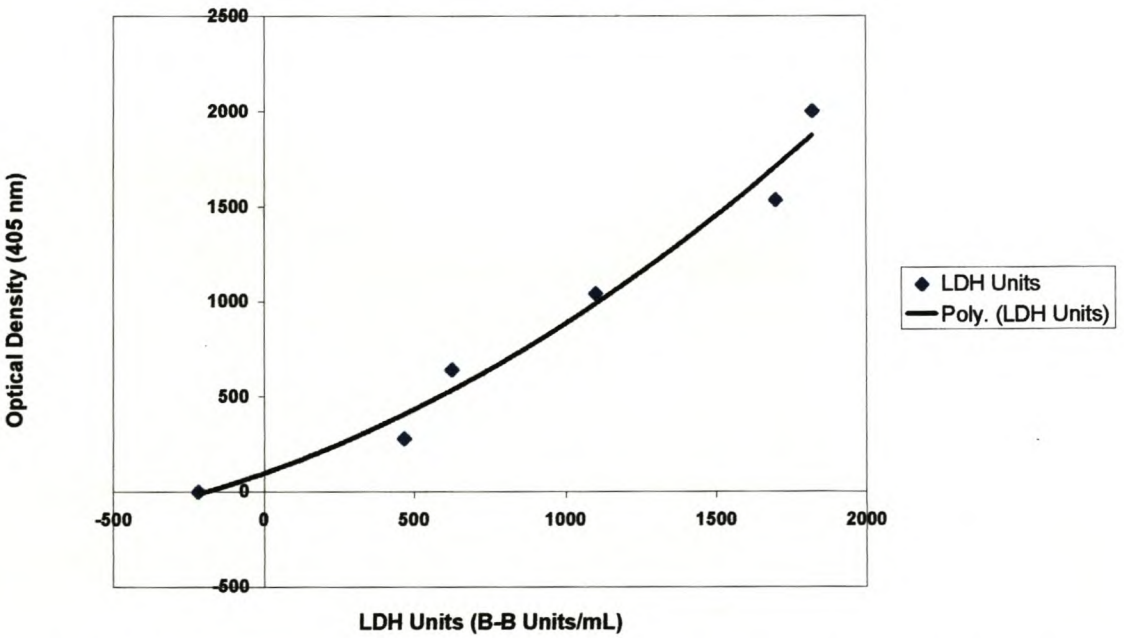

Figure 4.3 LDH standard curve for BALB/c mice as obtained from standards prepared in the laboratory. Poly. (LDH Units) represents the trendline observed for the amount of LDH present in the medium

Results summarised in Table 4.3 show that no LDH was secreted by shrew splenocytes exposed to varius concentrations of Cd into the culture medium after the two hour incubation period (Figure 4.4). The effects of Pb and Zn were not assessed by the LDH assay since one only wanted to determine whether the LDH assay was able to detect any changes to cell membrane integrity and therefore cell viability.

Table 4.3 Amount of LDH (B-B Units/mL) present in shrew splenocytes measured at 405nm from the LDH assays of shrew splenocyte cultures incubated at 37°C and treated with 0μM or 2μM of $CdCl_2$

LDH Cd 0μM	LDH Cd 2μM
-61.35	-47.46
-2.364	-25.61

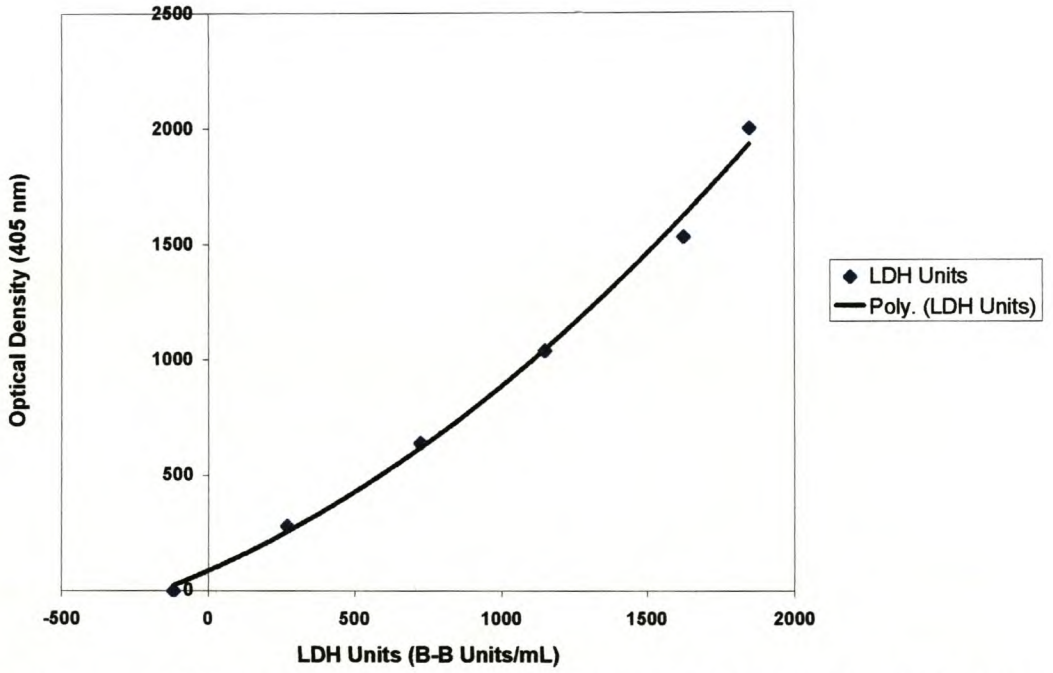

Figure 4.4 LDH standard curve for shrew data as obtained from standards prepared in the laboratory. Poly. (LDH Units) represents the trendline observed for the amount of LDH present in the medium

4.3.2 Cell Viability (Trypan Blue Exclusion) – Preliminary study

Figure 4.5 shows the percentage of viable mouse whole blood cells present in the culture medium after the cells were exposed to 20μM of Cd, Pb and $ZnCl_2$ for two hours at 37°C.

Incubation of the cells with no metal salts decreases the viability of the cells compared to the cell viability before incubation took takes place. This decrease in cell viability of the control cells is however less than that of the metal exposed cells and is not statistically significant ($p>0.05$). Whole blood cells that were exposed to 20μM Zn display a higher cell viability than that of the control cells. This increased cell viability of the Zn-exposed cells is also higher than the cell viability of the unexposed cells prior to incubation with the metal salts.

A decrease in the viability of the control cells of mice one to three was observed when compared to the cell viability of the original, unexposed cells before incubation with the metal salts. The cell viability of the unexposed cells before incubation (original cells) was taken to be 100%. This decreased viability displayed by the control cells in comparison to the cell viability of the original cells, was statistically significant ($p<0.05$).

For mouse four, there was a 10.25% increase in the viability of the cells from the control culture after the incubation period. This increase in cell viability was not statistically significant ($p>0.05$) (Table 4.4).

When comparing the cell viability of the Cd-exposed cells to the original viability count, there was a statistically significant decrease in the viability of the Cd-exposed cells of mice one to four ($p<0.05$).

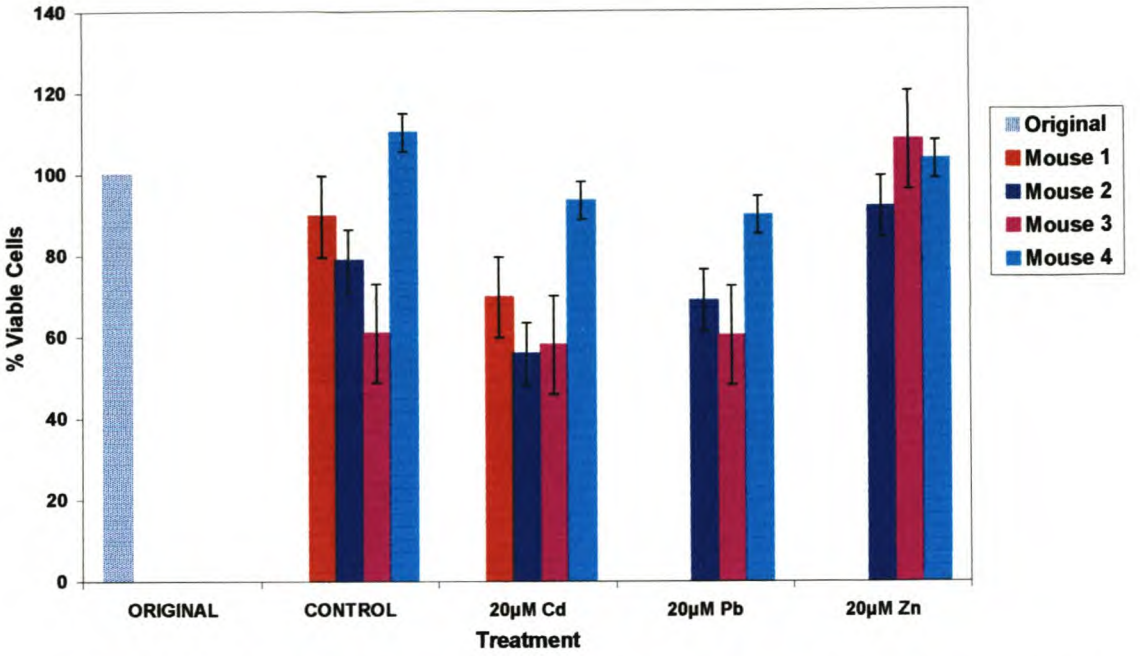

Figure 4.5 Percentage of viable mouse whole blood cells present in the culture medium after exposure of the cells to 20µM of Cd, Pb and Zn for 2 hours at 37°C. n=12

Table 4.4 shows the percentage of viable cells of the mouse whole blood cells present in the culture medium after the cells were exposed to 20µM of Cd, Pb and $ZnCl_2$ for two hours at 37°C.

The Zn-exposed cells of mouse two displayed a decrease in viability in comparison to the original viability count of the unexposed cells before incubation. This decreased viability was not however statistically significant ($p>0.05$). An increase in the cell viability of the Zn-exposed cells of mice three and four in comparison to the cell viability of the original cells was observed. The increased viability of the Zn-exposed cells of mouse four compared to that of the original cells was not statistically significant ($p>0.05$) though.

The increased cell viability of the Zn-exposed cells of mice two and three compared to the control viability counts was statistically significant ($p<0.05$). The Zn-exposed cells of mouse four displayed a decrease in cell viability when compared to the control viability counts. This decreased viability was not statistically significant ($p>0.05$).

When comparing the viability of the Cd-exposed cells of mice one to four with the viability counts obtained from the control treatments, there was an overall decrease. This decrease was statistically significant only for mice one, two and four ($p<0.05$). The slight

decrease in viability of the cells of mouse four exposed to Cd compared to the viability values of the control cells, was not statistically significant ($p>0.05$).

The Pb-exposed cells of mice two to four displayed a statistically significant decrease ($p<0.05$) in cell viability compared to the viability of the original cells. Whole blood cells obtained from mouse one were not exposed to Pb. For mice two to four a decreased viability of the cells exposed to 20µM of Pb in comparison to the viability count of the control cells was observed. The decrease in cell viability of the Pb-exposed cells of mouse three compared to the viability of the cells of the control cells was not statistically significant ($p>0.05$). The decreased viability observed in the cells of mice two and four when compared to the control viability counts, was statistically significant ($p<0.05$).

Table 4.4 Percentage of viable mouse whole blood cells present in the culture medium after exposure of the cells to 20µM of Cd, Pb and Zn for 2 hours at 37°C

Individual #	Original	Control	Cd	Pb	Zn
1	100	89.64	69.72	ND	ND
2	100	78.82	55.88	68.82	91.76
3	100	61.00	58.00	60.33	108.00
4	100	110.25	93.44	89.75	103.28

ND – Not Determined

Original - % viable cells counted before the cells were exposed to the metals

Figure 4.6 shows the results obtained from the dilution curve experiment with the various concentrations of Zn (0; 2.5; 5; 10 and 20µM Zn), showed that Zn increases the viability of the cells after the two hour incubation period. There is a sharp increase in the viability of the cells that is observed between the 5 and 10µM Zn range. Between the 5 and 20µM Zn concentrations the viability of the cells is increased above the 100% (original count) level.

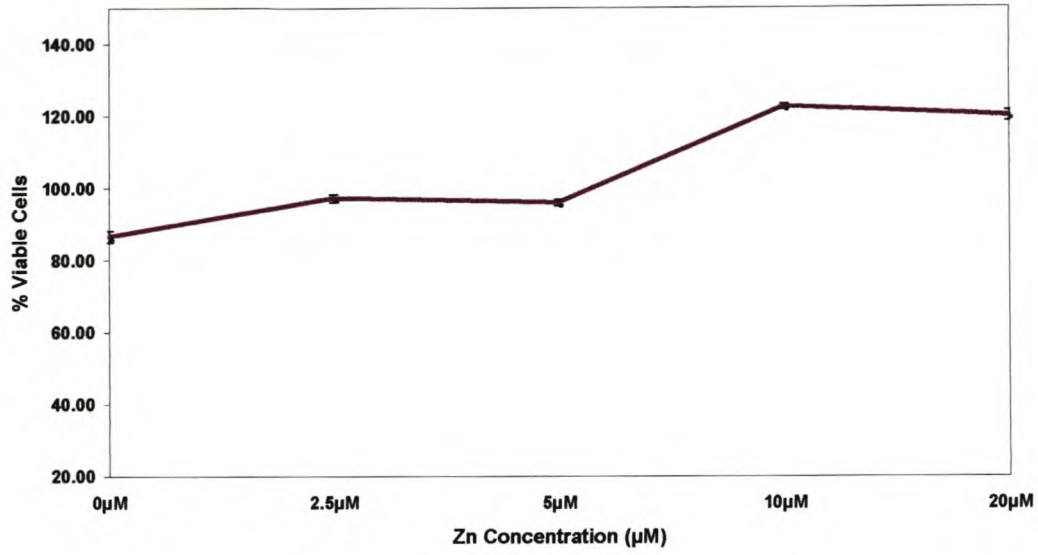

Figure 4.6 Percentage of viable cells mouse whole blood cells present in the culture medium after the cells were exposed to various concentrations of Zn for 2 hours at 37°C. n=12

4.3.3 Cell Viability (Trypan Blue Exclusion)

Figure 4.7 represents the cell viability of mouse whole blood cells that were exposed to Cd, Pb and Zn at concentrations of 8, 16, 20, 40, 80 and 100µM for two hours at 37°C. Letterlike symbols indicate statistical differences. This viability is relative to the control cells' viability. *P* values smaller than 0.05 were considered statistically significant. There was no statistically significant ($p>0.05$) difference in cell viability between Cd and Pb-exposed mouse whole blood cells in all of the exposure concentrations. There was no statistically significant difference ($p>0.05$) in cell viability between the control and Cd and Pb-exposed mouse whole blood cells for all of the exposure concentrations. There was a statistically significant difference ($p<0.05$) in cell viability between the control cells and the mouse whole blood cells exposed to 16µM and 100µM Zn. There was a statistically significant ($p<0.05$) difference in cell viability between mouse whole blood cells exposed to 8µM, 80µM and 100µM of Cd and Zn. There was a statistically significant difference ($p<0.05$) in cell viability between mouse whole blood cells exposed to 8µM, 20µM, 40µM, 80µM and 100µM of Pb and Zn.

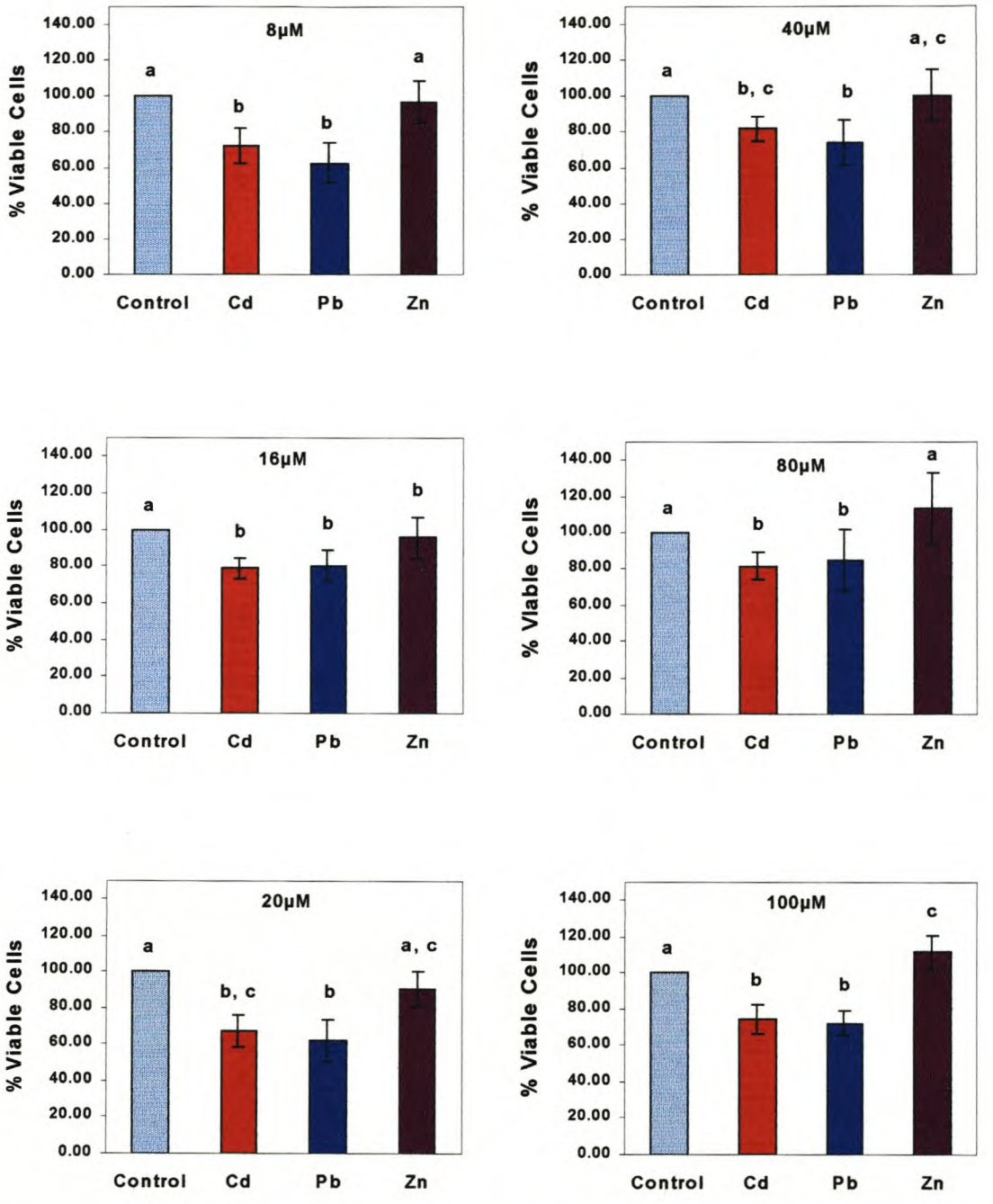

Figure 4.7 The Percentage of viable mouse whole blood cells after exposure to various concentrations of Cd, Pb and Zn for 2 hours at 37°C. Letters above the bars indicate statistically significant differences ($p<0.05$) (different letters) and not statistically significant (same letters) percentages. n=12

Figure 4.8 represents the mean percentage of viable cells of whole blood cells exposed to Cd, Pb and Zn at various concentrations ranging from 0, 8, 16, 20, 40, 80 and 100µM for two hours. This cell viability is relative to the viability of the control cells'.

The mouse whole blood cells that were exposed to 8, 16 and 20µM of Zn had lower cell viability than the control cells. The decreased cell viability of the cells that were exposed to 16µM of Zn, was statistically significant ($p<0.05$) from the cell viability of the control cells. Mouse whole blood cells that were exposed to Zn concentrations of 20, 40, 80 and 100µM displayed increased cell viability. This was not statistically significant however ($p>0.05$). The percentage of viable mouse whole blood cells that were exposed to 100µM of Zn was lower than that of the cells that were exposed to 80µM. This increase in the percentage of viable cells was statistically significant ($p<0.05$) from the control cells.

The Cd-exposed whole blood cells displayed a decrease in the percentage of viable cells at all the exposure concentrations. This decreased cell viability was statistically significant ($p<0.05$) from the control cells.

The Pb-exposed whole blood cells displayed a statistically significant ($p<0.05$) decrease in cell viability from that of the control mice.

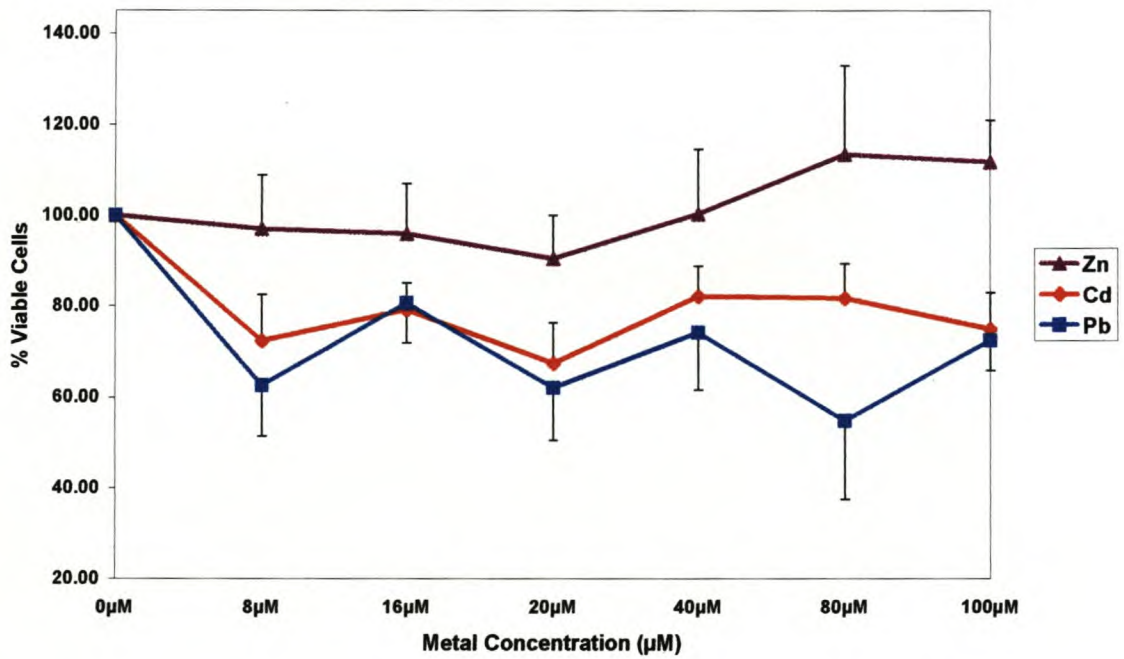

Figure 4.8 The viability of mouse whole blood cells after exposure of the cells to 0, 8, 16, 20, 40, 80 and 100µM of Cd, Pb and Zn for 2 hours at 37°C. Vertical bars indicate standard errors of the mean. n=12

4.3.4 DNA Determination

Table 4.5 summarises the results of the DNA synthesis experiment using *in vitro* incorporation of [^{3}H] Thymidine into the blood cells of the mice as a measure of DNA synthesis. No trend could be observed. The shrew DNA synthesis experiment was based only on one shrew. The data are too few to make any meaningful study of it.

Table 4.5 Percentage decrease of DNA content of mouse blood cells from whole blood cultures, compared to the control group after 2 hours of incubation at 37°C and treated with 0μM or 20μM of $CdCl_2$, $PbCl_2$, or $ZnCl_2$. The negative values indicate increased DNA content in the mouse whole blood cells

Individual #	Control	Cd	Pb	Zn
1	100	25.01	ND	ND
2	100	71.80	70.02	80.52
3	100	11.64	0.02	-6.16
4	100	-37.39	-67.70	-63.86

4.4 Discussion

The results obtained from the LDH assays performed on mouse and shrew splenocytes indicate that there was no increased LDH activity in the culture medium (Tables 4.2 and 4.3) i.e. there was no LDH being secreted into the culture medium after the mouse splenocytes were exposed to 20µM of Cd, Pb and Zn and the shrew splenocytes to 2µM of Cd for two hours at 37°C.

According to Butler (1996), LDH leaks from damaged cell membranes of non-viable cells into the extracellular pool. It therefore seems plausible that at the concentrations to which the splenocytes were exposed in this study, that there was no effect on the integrity of the cell membranes. However, studies by MacDonald, Simpson & Nossal (1987) have shown that heparin destabilises LDH and in the present study, the culture medium contained heparin to prevent blood clotting. The cells were cultured in this heparinised medium. LDH that possibly leaked from the damaged cell membranes, as a result of exposure of the cells to the metals could have been changed during the course of culture then as a result of the heparin in the medium. De Ruiter and Mailänder (1985) who also studied the effects of heavy metals on (various cellular parameters in Chinese hamster kidney cells) showed that Cd induced LDH leakage from cells exposed to the metal in concentrations as low as 0.01µM and 0.0001µM. These were much lower concentrations that those used for exposure during the present study. Hassoun & Stohs (1996) studied the effects of Cd on cell viability in the macrophage line J774A.1 and found that LDH activity increased linearly between 0.3µM and 0.7µM Cd. These studies therefore indicate that LDH leakage should have taken place during the present study, at the exposure concentrations used. The activity of the leaked LDH into the medium has most probably been masked by the destabilising action of the heparin on the enzyme. The results of the present study can therefore not be seen as conclusive as to the effect of the metals Cd, Pb or Zn on the cells.

In the present study, Cd and Pb had a greater cytotoxic effect than Zn after two hours of exposure with the mouse whole blood cells at all the concentrations to which the cells were exposed (Figures 4.5 and 4.7). The results of the preliminary study (Figure 4.6) measuring cell viability with the trypan blue exclusion assay, indicated that Cd and Pb have disrupting influences on the viability of the mouse whole blood cells exposed to 20µM of the metals Cd and Pb after short-term (two hours) exposure of the cells to these metals.

The results that were obtained after further investigating the effects of Cd, Pb and Zn at higher (and lower) concentrations than those used in the preliminary study, confirm that Cd and Pb decrease the viability of the cells and in doing so, negatively effect cell membrane integrity (Figures 4.7 and 4.8). These findings are supported by the study of Steffensen *et al.* (1994) who found that Cd and Pb are more cytotoxic to cell membranes than Zn.

In the present study, Zn statistically significantly increased the cell viability of the Zn-exposed cells at concentrations even higher than that used in the preliminary study (Figures 4.6 and 4.8). This is in contrast to the findings of Steinebach & Wolterbreek (1993), who found that Zn concentrations exceeding 50μM induced LDH leakage from rat hepatocytes and hepatoma tissue cultures.

The cytotoxicity of Cd and Pb has been established in a number of studies (Steffensen *et al.* 1994; Koropatnick & Zalups 1997). In a study performed by Steffensen *et al.* (1994), Zn and Cd had no effect on the viability of the human immune cells that were exposed to these metals, regardless of the concentrations (Zn 0 – 5 000μM and Cd 0 – 10 000μM) for the first 17-19 hours of incubation. A weak cytotoxic effect was observed for cells that were exposed to 1 000μM of Pb after 3-4 days of exposure. Steffensen *et al.* (1994) expressed "cytotoxic effect" in terms of the percentage of viable cells.

According to Goering, Mistry & Fowler (1987) and Segner & Braunbeck (1998), toxic metals enter cells after having interacted with the plasma membrane. Cell membranes are the sites of uptake, deposition and elimination of toxicants (Segner & Braunbeck 1998) and are also the sites where toxic effects are first noticed (Pritchard 1979). The cell membrane is the outer boundary of the cell. In order to enter the cell, toxicants and other substances need to pass through the cell membranes. According to Segner & Braunbeck (1998), special and facilitated transport, endocytosis or passive diffusion achieves this.

Cell membranes possess selective permeability to substances passing through it and are made up of proteins and a bi-lipid layer (mostly phospholipids). The proteins extend throughout the membrane. The phospholipds have polar "head" (hydrophilic) and non-polar "tail" (hydrophobic) regions. The hydrophobic part of the lipids are embedded within the membrane interior (Ganong 1995a).

Figure 4.9 is a schematic representation of a generalised cell membrane showing passive permeability pathways. The hydrophilic portions of the cell membrane contain functional groups. These groups regulate passive and carrier-mediated permeability pathways. Passive pathways depend on the electrochemical gradient to energise it's passage across the membrane and is not dependant on binding to specific molecules. Carrier mediated

pathways are either dependant on energy or on a carrier substance to carry substances across cell membranes (Pritchard 1979).

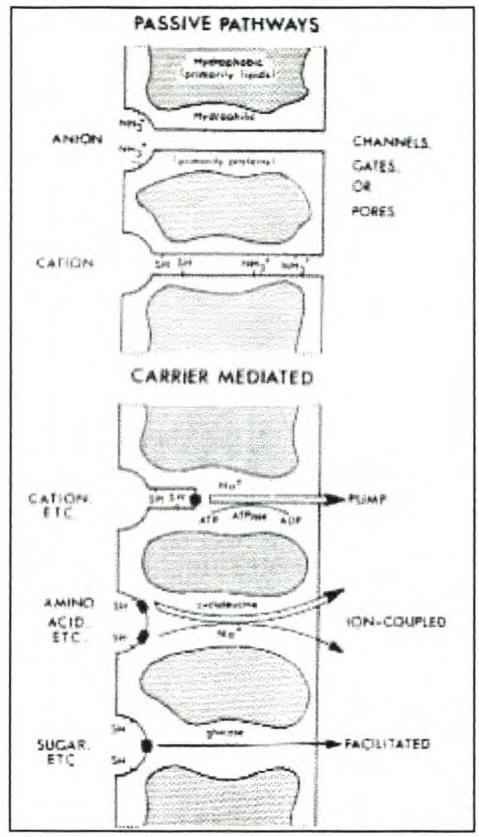

Figure 4.9 Schematic diagram of a generalised cell membrane showing permeability pathways. Hydrophobic portions (stippled) are in the interior; hydrophilic portions (unstippled) constitute the interface between the membrane and aqueous media. Thick arrows represent active transport and thin arrows indicate the movement of substances down an electrochemical gradient (adapted from Pritchard 1979)

Cell membrane processes (Figure 4.9) that are affected by xenobiotic toxicity include passive ion permeability; ion transport; competitive inhibition and Na^+ coupled intestinal sugar transport (Pritchard 1979).

Cadmium is carried across cell membranes via Ca^{2+} channels during facilitated transport (Segner & Braunbeck 1998). It not only interacts with phospholipids situated in the cell membrane, but also competes with Zn for sulfhydryl groups (Vallee & Ulmer 1972) situated on the cell membrane. Pritchard (1979) reports that Benesch & Benesch in 1954

suggested that modification of membrane sulfhydryl groups lead to changes in membrane permeability. This was confirmed by Rothstein in 1959, Rothstein, Takeshita & Knauf in 1972 and Grinstein & Rothstein in 1978.

Lead enters the cell via either passive diffusion or by binding to the membrane, after which endocytosis follows (Goering *et al.* 1987). Pb increases the fragility of the erythrocyte membranes and binds to phosphates in the cell membrane *in vitro*. It decreases the selective permeability of the membrane and decreases the activity of Na^+/K^+ATPase by being responsible for the movement of K^+ ions out of the cell. (Vallee & Ulmer 1972). The enzyme Na^+/K^+ATPase controls the transport of Na^+ ions out of and K^+ ions into the cell. The net movement of positive charge that the movement of the two ions produce, is required for the transport of essential substances (e.g. glucose transport into intestinal mucosal cells) in the vertebrate body (Ganong 1995a).

Very little is known about the mechanisms of Zn transport in the blood and the uptake of Zn by cells (Harris 1991; Steinebach & Wolterbreek 1993).

The increased cell viability of the Zn-exposed cells that were observed in the present study suggests that Zn might have the ability to stabilise cell membranes and prevent the loss of membrane integrity. For this to be possible there would have to have been an increase in the number of viable cells present in the culture medium and this is only possible if cell proliferation took place.

The stabilising effect of Zn on the cell membranes observed in the present study is supported by the findings of Chvapil (1973) and Bettger & O'Dell (1981). Bettger & O'Dell (1981), while studying the physiological role of Zn in the structure and function of membranes, found that Zn is very important to cell membrane integrity. These authors state that cell membranes become defective when cells lack Zn. When cell membranes become defective, cell growth and proliferation are inhibited (Steinebach & Wolterbreek 1993) and the cells become unstable. Ions leak across membranes, peroxidation can occur and cell death can follow. Certain toxicants can also enter the cell as a result of the decrease in selective permeability of the cell membrane. Chvapil (1973) proposed that Zn stabilise cell membranes by altering the fluidity and stability of plasma and lysosomal membranes.

Zinc is described as a mitogenic substance by Golden (1989), i.e.; Zn is capable of stimulating mitosis, the process whereby cells divide (Golden 1989). These authors found that mitotic cells could be observed as soon as two to four hours after incubation of the cells with a variety of toxicants. This would be responsible for an increase in the number of cells present in the culture and would account for the viability increases above 100% that of the

control cells that was observed in cells exposed Zn exceeding 20µM. Because Zn is able to stabilise cell membranes, cells with damaged membranes that would not ordinarily have been able to divide, are now able to undergo mitosis since the integrity of their cell membranes have been restored (cell membrane integrity is required for normal functioning of cells). The results of the present study of mouse whole blood cells exposed to Zn confirm this membrane stabilising effect for Zn on cell membranes.

In a normal functioning cell, DNA is constantly being broken down and repaired and synthesised (Clegg, Keen & Hurley 1989). DNA synthesis is an important metabolic function without which the cell cannot survive (Wilson 1992). According to Ecobichon (1992), unscheduled DNA synthesis is indicative of functional DNA repair mechanisms and will result in the incorporation of the DNA precursor Thymidine. The results of this study show that no trend could be observed however regarding the effect of Cd, Pb and Zn on DNA synthesis in either the shrew or BALB/c mice. A further study was therefore done to determine possible DNA damage using a different method (Refer to Chapter 5).

Chapter 5

DNA Damage in Mouse Whole Blood Cells after Exposure to Cd, Pb and Zn

5.1 Introduction

Deoxyribonucleic acid (DNA) is present in the nuclei of living cells of eukariotic organisms as a functionally stable double stranded helix without discontinuity (Ganong 1995a). In normal, healthy cells it has no abnormal structural modifications (adducts or deviant base pairing) and in this state it has a high degree of integrity (Shugart 1998). DNA is the component of chromosomes that carries the genetic message, the blue print of life for all heritable characteristics of cells and their descendants, and its integrity is therefor vital to the survival of organisms.

DNA is continuously being subjected to spontaneous chemical degradation that might interfere with its integrity and functioning as the carrier of genetic material (Lindahl 1993). In order for organisms to survive, the integrity of DNA has to be maintained rigidly (Eastman & Barry 1992; Shugart 1998). Therefor specific DNA repair processes have evolved to counteract the effects of hydrolysis, oxidation, the non-enzymatic methylation of DNA, as well as chemical and radiation induced DNA damage (Lindahl 1993).

Chemicals that can modify the structure of DNA and adversely affect its integrity (structure or function) are termed genotoxicants (Shugart 1998). Some genotoxicants of anthropogenic origin are known carcinogens (the effects lead to neoplastic changes) and mutagens (the effects lead to heritable changes) (Ballantyne, Marrs & Turner 1993). Exposure of an organism to genotoxicants results in the expression of various biological responses e.g. DNA adduct formation, DNA strand breaks, DNA repair, base modification and chromosomal aberrations (Shugart 1998). The spontaneous chemical degradation to which DNA is naturally subjected also cause changes to the structure of DNA (Eastman & Barry 1992).

The presence of DNA strand breaks in cells is not uncommon and most cells are able to rapidly repair these structural changes in DNA (Carson, Seto, Bruce-Wasson & Carrera 1986; Shugart 1998). Breaks in DNA are caused by the loss of a base from the DNA molecule and are characterised by gaps in the molecule in an attempt by the cell to repair the damage (Shugart 1998). According to Eastman & Barry (1992), DNA breaks are categorised as either single or double stranded breaks.

Single strand breaks are repairable lesions in which there are gaps in one of the strands that form the double helix. They are not considered to be significantly lethal mutagenic lesions (Collins, Dobson, Dušinská, Kennedy & Štětina 1997) because most of them can be rapidly repaired (Halliwell & Aruoma 1991; Eastman & Barry 1992; Collins *et al.* 1997) by religation or dephosphorylation before religation. Others require a more complicated repair process but are still repairable in a fairly short time.

Double strand breaks are usually considered lethal because they contain gaps in both strands and the two correct ends are unlikely to religate (Eastman & Barry 1992). They are considered to be the primary lesions that are responsible for cell lethality (Tice 1995) since double strand breaks are potentially responsible for the loss of base sequence information. Repair of these double stranded breaks does occur, but is usually more complex than in single strand breaks and more often that not lead to base sequence errors and chromosomal abberations (Eastman & Barry 1992; Ward 1994).

Chromosomal or DNA damage is generally accepted to be indicative of mutagenic or carcinogenic effects (Fairbairn, Olive, & O'Neill 1995). According to Fairbairn *et al.* (1995), such effects to the structure of DNA are the result of long-term exposure to genotoxicants. In short-term genotoxicity assays, the most common endpoints that are measured include DNA damage, point mutations and chromosomal aberrations. But measuring these endpoints *in vitro*, do not always produce positive results that correlate with the findings of *in vivo* assay results.

Damage to DNA has been proposed as a useful biomarker of genotoxicant exposure (Shugart 1998). According to Weeks (1995), a biomarker is a biological response to a chemical or chemicals and give a measure of exposure and sometimes also of toxic effect. Such effects may be measured at the biochemical or molecular level (Van Gestel and Van Brummelen 1996; Theodorakis & Shugart 1998) and can be manifested as changes to macromolecular structure and function (Theodorakis & Shugart 1998) e.g. changes to DNA structure.

Shaw (1998) states that DNA biomarkers are at an advantage over traditional measures of environmental toxicant exposure because toxicant bioavailabilty is accounted for and the techniques that are employed to measure DNA damage are sensitive and do not necessarily require that the organisms be destroyed.

According to Eastman & Barry (1992), McKelvey-Martin, Green, Schmezer, Pool-Zobel, de Méo & Collins (1993) and Shugart (1998), various methods exist for measuring DNA strand breaks. These include the Alkaline elution technique, DNA precipitation, alkali unwinding and gel electrophoresis. The basic principles for determining strand breaks in DNA remain the same in the various assays (Eastman & Barry 1992) and limits, advantages and disadvantages in every assay exist (McKelvey-Martin *et al.* 1993; Shugart 1998). The differences between the assays lie in either the manipulation of the DNA preparation before the release of the single stranded DNA or in the method of detecting breaks in the DNA molecule (single or double strand breaks) (Shugart 1998).

Prior to 1975, the most common technique that was employed to measure DNA strand breaks was the alkaline sucrose gradient centrifugation assay (Eastman & Barry 1992). It involved the lysing of cells and the alkalic denaturing of DNA. The single stranded length of DNA was calculated from the distance sedimented along the test tube. Alkali-labile sites were converted to breaks that were detected by this technique. This technique however, did not distinguish between the two types of DNA strand breaks.

Alkaline elution analysis was substituted for the centrifugation technique. It could be applied to more samples than the centrifugation technique, and it could resolve DNA of a much higher molecular weight. The alkaline elution technique is based on the principle that damaged DNA is eluted faster than undamaged DNA and the rate of elution is proportional to the single strand size of DNA. Variation of this technique by neutral elution analysis makes it possible to distinguish between single and double strand breaks. Neutral elution detects double strand breaks (Eastman & Barry 1992).

The DNA precipitation assay was developed by Olive in 1988. This assay makes it possible to quantify the amount of the two types of DNA breaks. Denatured single stranded DNA is released from a physical matrix of cellular proteins. This assay allows the separation of single stranded DNA from double stranded DNA during denaturation.

Rydberg & Johanssen (1978) were the first to quantify DNA damage in individual cells. The cells were embedded in agarose on slides (microgels) and lysed under mild alkaline conditions. This enabled the partial unwinding of DNA. After neutralisation, the cells were stained with a fluorescent, DNA binding dye and the extent of DNA damage was

quantified. The ratio of double (green cells) to single stranded (red cells) DNA was measured (Tice 1995). According to Shugart, Bickham, Jackim, McMahon, Ridley, Stein & Steinert (1989b), the Alkaline unwinding assay is a sensitive method for detecting strands breaks in DNA.

The Comet assay or Single cell gel electrophoresis assay is a simple sensitive, rapid and visual microgel technique whereby DNA damage is measured cell by cell (McKelvey-Martin-Martin *et al.* 1993; Fairbairn *et al.* 1995; Collins *et al.* 1997). This technique was first described by Ostling and Johanson (1984) (Olive, Wlodek & Banath 1991a; Fairbairn *et al.* 1995; Tice 1995). It involves embedding the cells in agarose on slides, lysing the cells under mild alkali conditions and electropherising the DNA under neutral conditions (Fairbairn *et al.* 1995; Tice 1995). The method as described by Ostling and Johanson in (1984) only detects double strand breaks (Tice 1995). The term "comet" resulted from the images of the cells that were viewed under a fluorescent microscope.

Singh, McCoy, Tice & Schneider (1988) modified the Comet assay to be able to detect single strand breaks by electrophoresing DNA under strong alkali conditions. This modified form of Ostling and Johanson's Comet assay was first referred to by Singh and his colleagues as the Single Cell Gel Electrophoresis assay but many authors also refer to this method as the Comet assay (Tice 1995). Since 1988, Olive and co-workers developed versions of the 1984 neutral electrophoresis technique that involves the alkali lysis of DNA, that enables the detection of single strand breaks (Olive *et al.* 1991; Tice 1995) not previously possible under neutral lysis conditions employed by Ostling & Johanson (1984). See Figure 5.1 for a general protocol for both the alkaline and neutral assays.

DNA within the nucleus is found as looped supercoils and the principles of detection of the Comet assay are firmly rooted in the ability of DNA strand breaks to decrease the size of the DNA molecule (Fairbairn *et al.* 1995) by relaxing the supercoiled DNA (McKelvey-Martin *et al.* 1993; Collins *et al.* 1997). When DNA is broken down, the supercoils relax and a halo is formed around the nucleoid and the loops with the free loops extending outside of the nuclear matrix. Under high alkali conditions, DNA base pairing is disrupted and strands tend to separate. The unwinding of the DNA molecule occurs from the ends of the molecule and from internal single strand breaks (Collins *et al.* 1997).

Electrophoresing the DNA results in the electric current pulling the negatively charged DNA from the nucleus. Relaxed and broken DNA fragments from within the nucleus migrate further in the electric field. The resultant images, viewed using a fluorescent microscope,

resemble comets and it is from these images that the extent of DNA damage can be assessed (Fairbairn *et al.* 1995).

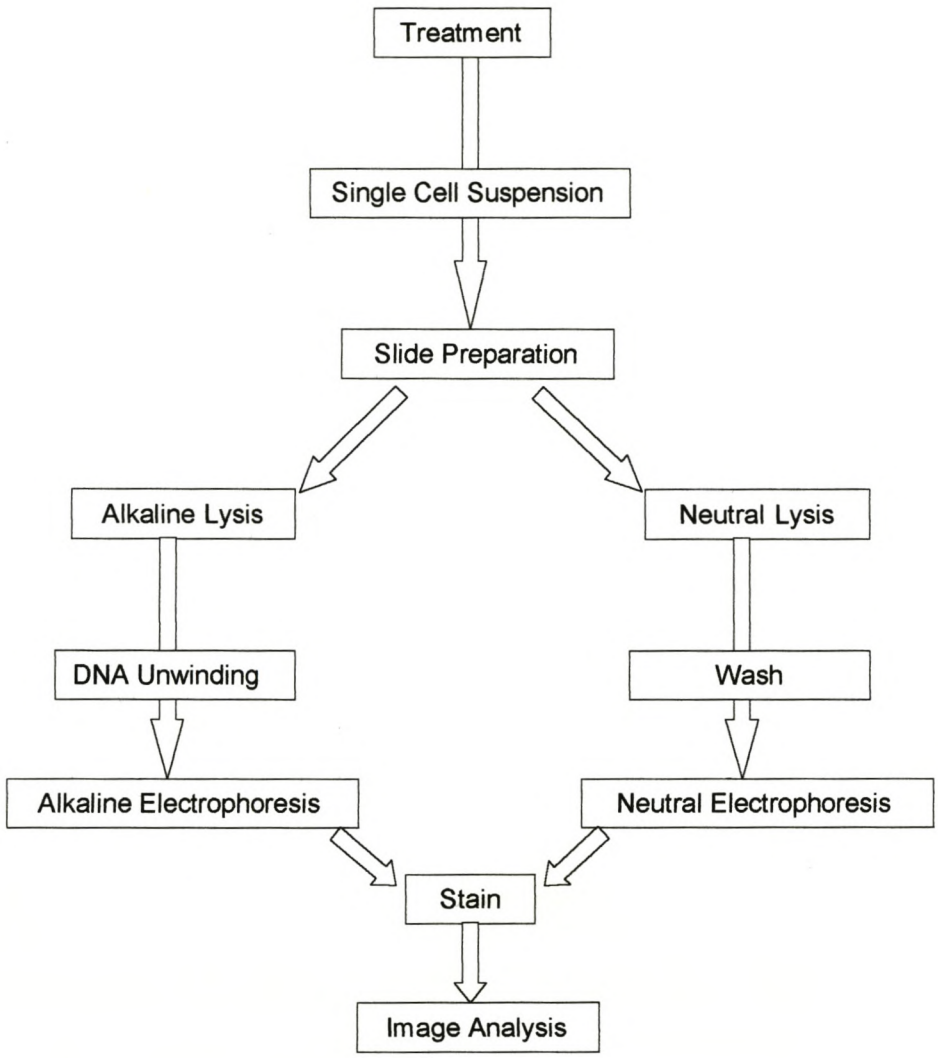

Figure 5.1 General schematic of the comet assay protocol. A general protocol for both the alkaline and neutral assays (adapted from Fairbairn *et al.* 1995)

The displacement of DNA from the nucleus is used as an indication of DNA damage (Olive *et al.* 1991). The head of the comet represents the intact, undamaged, supercoiled DNA while the tail consists of the relaxed loops of DNA that migrated out of the nucleus. The number of loops in the tail is representative of the number of DNA strand breaks induced by genotoxicants (Olive, Wlodeck, Durand & Banath 1992; Collins *et al.* 1997).

Either the tail length of the comet or the intensity of the DNA in the tail can be measured as DNA damage. The tail length and the intensity of DNA in the tail can also be combined quantitatively to obtain the tail moment (McKelvey-Martin *et al,* 1993; Fairbairn *et al,* 1995; Collins *et al,* 1997).

According to Vershaeve & Gilles (1995) and Schröder, Hassanein, Lauenroth, Koziol, Mohamed, Lacorn, Steinhart, Batel & Müller (1999), tail moment is the best way to quantify the amount of DNA damage. It was initially thought that tail length alone was sufficient to indicate DNA damage since higher degrees of damage resulted in more and smaller fragments of DNA migrating in the electric field during electrophoresis. This was visually confirmed by longer tails being observed with increasing levels of damage by genotoxicants. But it has recently been shown that, although tail length increases steadily for low levels of damage, a plateau is reached beyond a certain point. While tail length eventually reaches a plateau, the amount of DNA in the tail does not.

The comet assay has various applications. It has been used in *in vitro* and *in vivo* studies to assess DNA damage and repair induced by genotoxicants in a variety of mammalian cells (McKelvey-Martin *et al.* 1993). The comet assay is a biomarker of exposure and/or effect measuring DNA damage as the endpoint that specifically measures structural alterations to DNA that were caused by genotoxicants (Shugart *et al.* 1989b). It has also been used in biomonitoring studies and is especially useful for assessing genotoxic damage. The assay has also been used to distinguish irradiated from non-irradiated food (McKelvey-Martin *et al* 1993; Tice 1995).

The aims of the present study were to determine whether Cd, Pb and Zn effected damage to the structure of DNA of toxic metal exposed mouse whole blood cells *in vitro*. The comet assay, performed under alkali conditions (lysis and electrophoresis), was therefore employed to measure the DNA breaks in the whole blood cells of BALB/c mice as a measure of DNA damage.

5.2 Materials and Methods

5.2.1 Animals

Three female BALB/c mice were used in this study (See section 2.3.4.1)

5.2.2 Cell Preparation

Blood was obtained from the caudal vein of the mice as described in section 2.3.4.1.

5.2.3 Cell culture conditions

A cell density of $5x10^6$ cells/ml were used throughout this study for the whole blood cultures of the mice. 40µl of this volume of cells were pipetted (Eppendorf) into each well of a 96-well Nunclon multiplate (See sections 2.3.5.1).

5.2.4 Metal solutions

Metal ion ($CdCl_2$, $PbCl_2$ and $ZnCl_2$) stock solutions were made up in distilled water to a concentration of 10mM. This metal ion stock solution was then further diluted with RPMI 1640 medium before addition to the cell suspensions to give final concentrations of 20, 40, 80, 100, 150, 200, 250 and 500µM of the metal salts $CdCl_2$, $PbCl_2$ and $ZnCl_2$. 10µl of the metal salt solutions were added to each well of the 96-well Nunclon multiplate containing 40µl of cell suspension, except the blank wells. These received 10µl RPMI 1640 medium and represented the negative controls. The cell cultures were then incubated at 37°C for 2 hours in a Labcon growth chamber (See sections 2.3.6 and 2.3.7).

5.2.5 Comet Assay

Microscope slides (Chance Propper Ltd) were scoured along the edges with sandpaper to roughen them so that the gels would stay on the slides during lysis and electrophoresis. This is when the gels are more likely to slip off of the slides. The microscope slides were labelled with a diamond pen. 1% Normal melting point Agarose (NMA) was made up in Phosphate Buffered Saline (PBS) and the slides dipped in the molten NMA so that a thin layer of NMA coated the slide. The underside of the microscope slide was wiped clean and the gel layer was covered with a cover slip (Chance Propper Ltd). The slides were put on ice on a tin tray for the agarose gels to solidify.

The cover slips were removed when the gels were hard and the coated microscope slides stored at room temperature in a dust free container till they were needed.

Lysing solution was made up (2.5M NaCl, 100mM EDTA, 10mM Tris, pellitised NaOH) to a pH of 10.5. HCl and NaOH were used to manipulate the pH. Afterwards, 1% Triton X-100 and 10% DMSO were added. The solution was poured into Coplin jars and left overnight at 4°C.

A 0.5% Low melting point agarose solution (LMPA) in PBS was made up and 5ml samples aliquotted into polytops. The LMPA in polytops were then placed in a water bath to stabilise at 37°C.

A 5µl volume of the incubated cells (see sections 2.3.6 and 2.3.7) were mixed in an Eppendorf microfuge tube with 70µl of LMPA*. The cell suspension and LMPA were mixed in the microfuge tube by sucking up and expelling the mixture approximately 5 times with a micropipette (Costar). The cell/LMPA mixture was pipetted onto the coated microscope slides and the microscope slides again covered with cover slips. The microscope slides were placed on ice, in the dark till the new gel layer hardened. When the gels had hardened, the cover slips were removed and 75µl of LMPA pipetted onto the gels on the slides. This third layer was again covered with a cover slip and the slides placed on ice, in the dark for the new gel layer to solidify. The cover slips were again removed once the gel layer had hardened.

As controls, slides with cells treated with 0µM of metals (i.e. Cd, Pb or Zn) were at this stage either subjected to zero UV radiation (negative controls) or 120mJ.s^{-1} of UV radiation for 2.5 minutes using a cross linker (Ultra Lam, Scientific equipment) (positive controls).

After the cover slips were removed the slides were placed into the cold lysing solution in Coplin jars and left overnight (for at least 15 hours) at 4°C. After the lysing period the slides with microgels were removed from the lysing solution and placed horizontally on a staining rack over a sink. They were washed with distilled H_2O and drained by blotting the sides on tissue paper. After washing thoroughly, the slides were placed horizontally in a gelbox (Scigen) next to each other and as near as possible to the anode. Electrophoresis buffer (10N NaOH and 200mM EDTA) with alkali pH was poured over the microgels till a height not exceeding 0.25cm above the microgels (this level was important to ensure a specific voltage when electrophoresing) The microgels were left in the gelbox, in the dark, for 20 minutes to enable unwinding of the DNA.

* This and the following steps of the Comet Assay, were all performed in faint (semi-dark)light

After 20 minutes, the power was switched on and kept at a constant voltage of 25V and current of 300mA. The voltage and current were checked every 5 minutes and readjusted by adding or removing electrophoresis buffer when needed. Electrophoresis was allowed to continue for 20 minutes whereafter the power was turned off, the slides removed and washed with distilled H_2O on a staining rack. Thereafter the microgels were washed three times with neutralising buffer (0.4M Tris, pH 7.5), each time for 5 minutes. After the 3rd wash the slides were drained again, allowed to air dry, in the dark, away from dust and stored.

For observation and quantification of damage, microgels were stained with 20µg/ml of ethidiumbromide, drained and covered with a cover slip.

The individual cells were then scored for damage beneath a fluorescent microscope at the 25X objective, using an eyepiece micrometer, by measuring the tail length of each. At least 100 cells were scored on each slide. Refer to table 5.1 for a summary of the Comet assay protocol (alkali conditions) used in this study.

Four classes of damaged cells were identified, using the length of the tail of the comets. See table 5.2 for the various classes of damage and figure 5.2 for examples of what these cells would look like.

Table 5.1 Summary of the steps involved in the Comet Assay used in this study

Step #	To do
1	Scour slides with sandpaper
2	Prepare stock Lysing solution (alkali), store @ 25°C
3	Prepare 1% NMA in PBS
4	Dip slides into NMA
5	Cover with cover slip, place on ice till gels harden, remove cover slips
6	Store in dust free container
7	Make up metal solutions in RPMI 1640 medium
8	Obtain blood cells from mice
9	Suspend blood cells in RPMI 1640 medium
10	Seed 96-well plate with cell suspension and metal solutions
11	Culture *in vitro* metal-exposed cells @ 37°C, 2hrs
12	Prepare final Lysing solution (alkali), store @ 4°C
13	Prepare Electrophoresis Buffer (alkali), store @ 4°C
14	Prepare Neutralising Buffer, store @ 4°C
15	Prepare 0.5% LMPA in PBS
16	Remove cells from culture after 2hrs
17**	Mix 5µl cell suspension and 70µl LMPA
18	Drip onto NMA coated slide
19	Rpt step 5
20	Drip 75µl LMPA onto slide
21	Rpt step 5
22	Expose cells to UV ($120mJ.s^{-1}$, 2.5 min) if necessary
23	Place slides in Lysing solution, store overnight @ 4°C
24	Place gelbox on ice
25	Remove slides from Lysing solution, wash with dH_2O, blot dry
27	Place in gelbox, close to anode
28	Fill gelbox with Electro/P buffer till 0.25cm above slides
29	Leave in gelbox in dark, 20 min
30	Electrophorese @ 25V, 300mA, 20 min
31	Remove slides from gelbox , wash slides with dH_2O
33	Cover with Neutralising buffer, 5 min, Drain buffer on paper, Rpt 3x
34	Allow microgels to dry, store in dust free container
35	Stain with cells with $20\mu g.ml^{-1}$ ethidiumbromide, score cells

**This and the following steps are performed in faint (semi-dark) light

Table 5.2 Description of the classes of DNA damage in each of the tail length categories

Class	Description	Tail length interval (nm)
1	Undamaged	0-9
2	Very little damage	10-19
3	Damaged	20-29
4	Extensive damage	30+

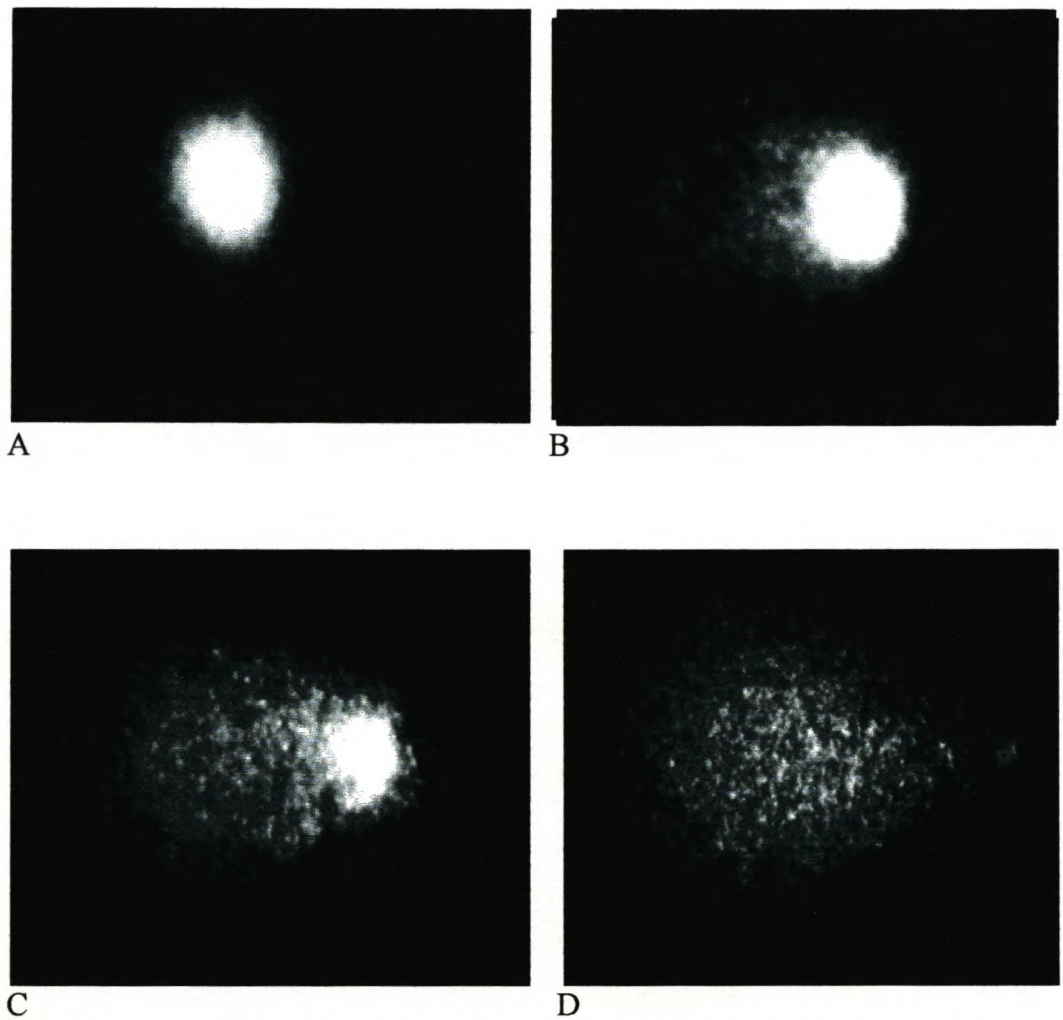

Figure 5.2 Examples of what damaged cells in the various classes of damage would look like. A: class 1; B: class 2; C: class 3; D: class 4 (Collins 2000)

5.3 Results

Figure 5.3 shows the spread of damage in the four classes of both the positive and negative control cells. The cells were not exposed to any metals, but were incubated only with RPMI 1640 medium for two hours at 37°C. Negative control cells were those that were not exposed to any UV radiation, while positive control cells were those that were exposed to 120mJ.s^{-1} for 2.5 minutes The cells that were not exposed to any UV radiation showed more DNA damage in all of the four classes of DNA damage, than those cells that were exposed to UV radiation. The n value refers to single cell numbers counted per treatment.

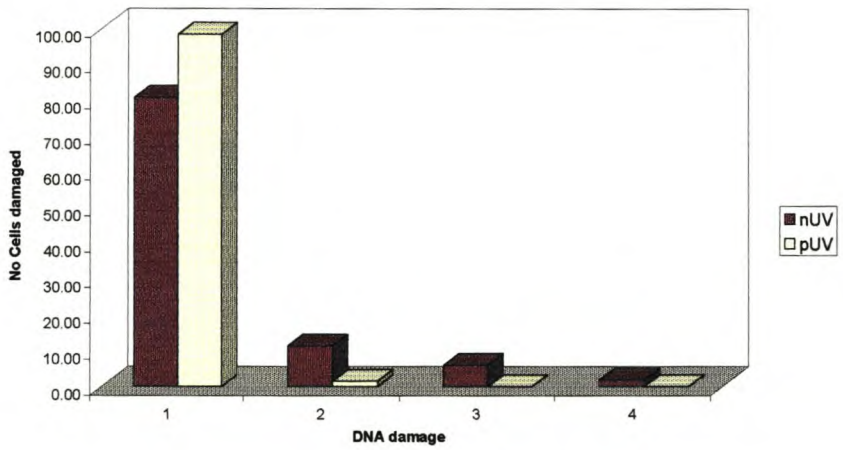

Figure 5.3 The number (No) of DNA damaged cells of the negative control (n=1 000) (0mJ.s^{-1} UV radiation) and positive control (n=200) (120mJ.s^{-1} UV radiation) treated cells in the different classes of DNA damage (1-4).

Figure 5.4 represents cells exposed to 20µM of Cd, Pb and Zn for two hours at 37°C. The Cd exposed cells showed the least amount of DNA damage, with 100% of the cells counted falling into the first class of DNA damage. Lead and Zn exposed cells showed DNA damage across classes 1 to 3 while Zn treated cells also showed class 4 DNA damage.

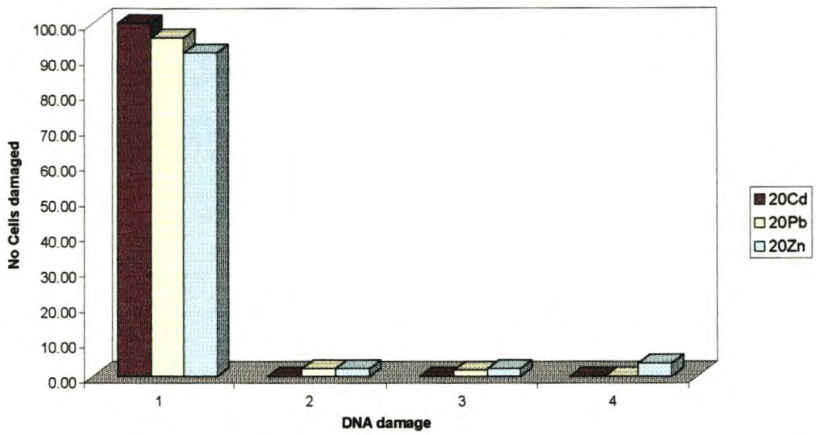

Figure 5.4 The number (No) of DNA damaged cells in each of the four classes of damage (1-4) of the cells exposed to 20µM of Cd (n=300), Pb (n=400) and Zn (n=400) for 2 hours at 37°C

Figure 5.5 represents cells exposed to 40µM of Cd, Pb and Zn for two hours at 37°C. The Cd exposed cells showed the least amount of DNA damage, with 100% of the cells counted falling into the first class of damage. Lead and Zn treated cells showed DNA damage across classes 1 to 4. Lead treated cells showed more DNA damage than Zn treated cells.

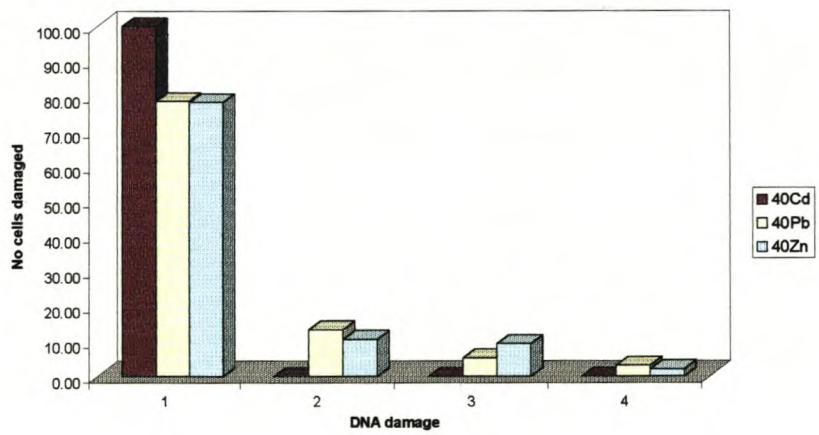

Figure 5.5 The number (No) of DNA damaged cells in each of the four classes of damage (1-4) of the cells exposed to 40µM of Cd (n=400), Pb (n=400) and Zn (n=400) for 2 hours at 37°C

Figure 5.6 represents cells exposed to 80µM of Cd, Pb and Zn for two hours at 37°C. The Cd exposed cells showed the least amount of DNA damage. Zinc exposed cells showed more DNA damage than the Pb exposed cells.

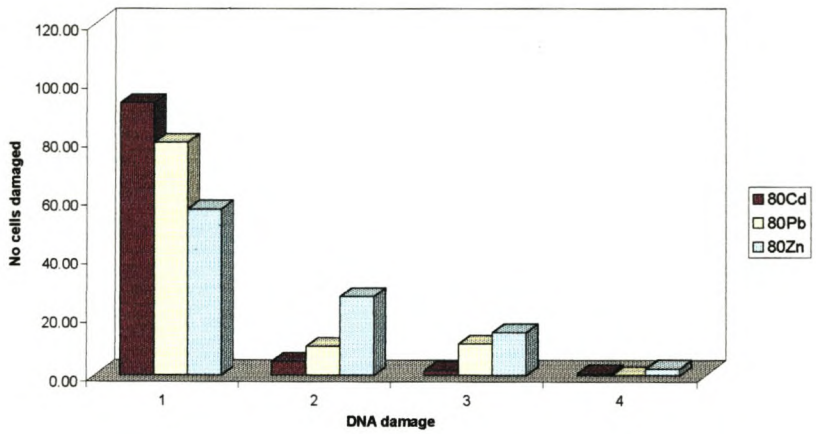

Figure 5.6 The number (No) of DNA damaged cells in each of the four classes of damage (1-4) of the cells exposed to 80µM of Cd (n=300), Pb (n=400) and Zn (n=400) for 2 hours at 37°C

Figure 5.7 represents cells exposed to 100µM of Cd, Pb and Zn for two hours at 37°C. The Cd exposed cells showed the least amount of DNA damage. The Zn exposed cells showed more DNA damage than the Pb exposed cells.

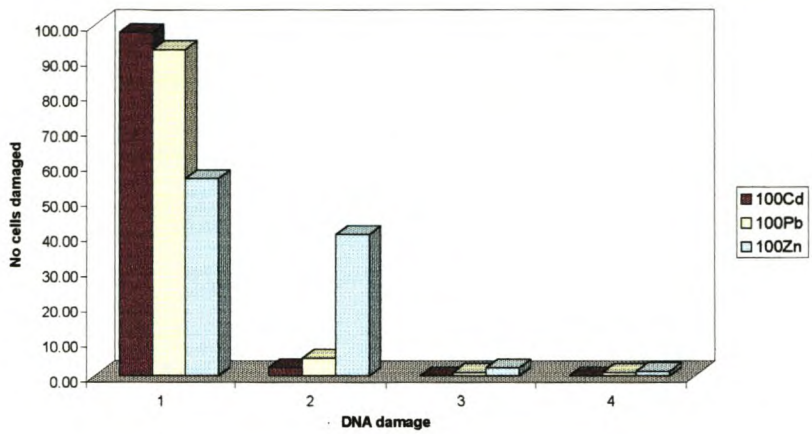

Figure 5.7 The number (No) of DNA damaged cells in each of the four classes of damage (1-4) of the cells exposed to 100µM of Cd (n=400), Pb (n=400) and Zn (n=400) for 2 hours at 37°C

Figure 5.8 represents cells exposed to 150µM of Cd, Pb and Zn for 2 hours at 37°C. The Cd exposed cells showed the least amount of DNA damage. Zinc exposed cells showed more DNA damage than the Pb exposed cells. There were more class 4 damaged Pb treated cells than Zn treated cells in the same class.

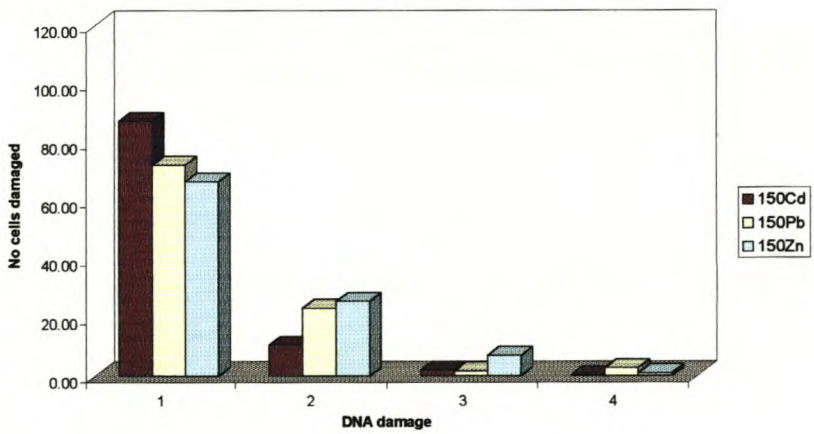

Figure 5.8 The number (No) of DNA damaged cells in each of the four classes of damage (1-4) of the cells exposed to 150µM of Cd (n=400), Pb (n=400) and Zn (n=400) for two hours at 37°C

Figure 5.9 represents cells exposed to 200µM of Cd, Pb and Zn for two hours at 37°C. There was a large number of cells from all three of the metal exposure groups that showed class 1 DNA damage. The Cd exposed cells showed the least amount of DNA damage. The Zn exposed cells showed less damage than the Pb exposed cells, with a high number of Pb treated cells showing class 3 DNA damage.

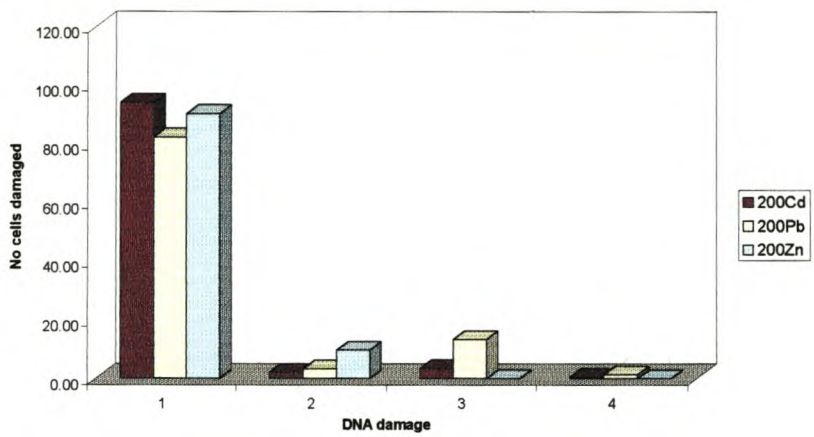

Figure 5.9 The number (No) of DNA damaged cells in each of the four classes of damage (1-4) of the cells exposed to 200µM of Cd (n=400), Pb (n=400) and Zn (n=400) for 2 hours at 37°C

Figure 5.10 represents cells exposed to 250µM of Cd, Pb and Zn for two hours at 37°C. A large number of cells from all three of the metal exposure groups showed very little DNA damage (class 1). The Zn exposed cells showed the least amount of DNA damage. The Cd exposed cells showed less damage than the Pb treated cells. The Pb exposed cells showed the most DNA damage.

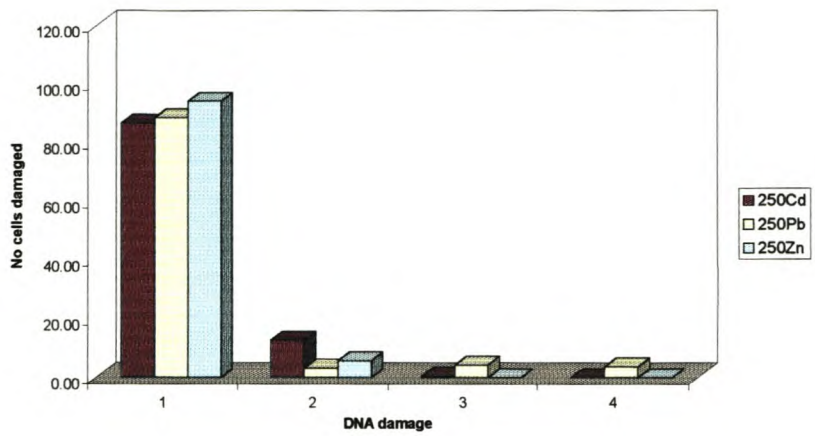

Figure 5.10 The number (No) of DNA damaged cells in each of the four classes of damage (1-4) of the cells exposed to 250µM of Cd (n=400), Pb (n=400) and Zn (n=400) for 2 hours at 37°C

Figure 5.11 represents cells exposed to 500µM of Cd, Pb and Zn for two hours at 37°C. A large number of Cd and Pb treated cells showed class 1 DNA damage. The Cd exposed cells showed the least amount of DNA damage. The Pb exposed cells showed less damage than the Zn exposed cells. The Zn exposed cells showed the most DNA damage.

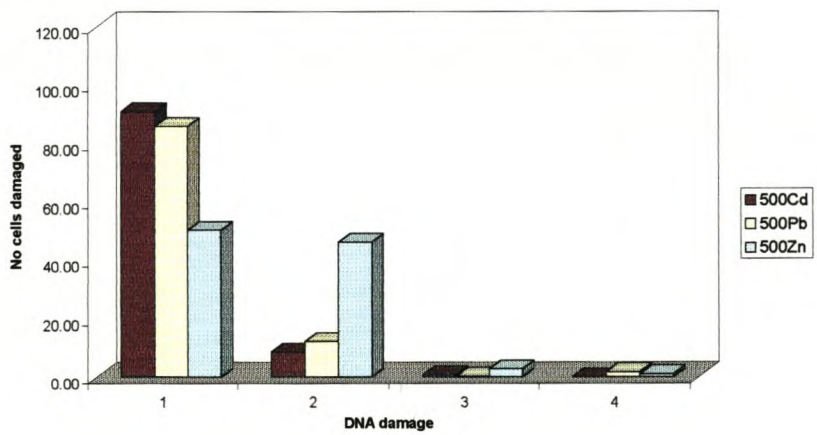

Figure 5.11 The number (No) of DNA damaged cells in each of the four classes of damage (1-4) of the cells exposed to 500µM of Cd (n=400), Pb (n=400) and Zn (n=400) for 2 hours at 37°C

5.4 Discussion

The results of the present study show that Cd, Pb and Zn caused structural damage to the DNA of the mouse cells. Cd had the least effect on DNA damage when compared to Pb and Zn (Figures 5.4-5.11). Zinc damages the DNA more than Pb (Figures 5.4, 5.6, 5.7, 5.8 and 5.11)

Zherbin, Chuklovin, Köteles, Kubasova, Vashchenko & Hanson (1986) studied the effects *in vitro* of Cd ions on membrane and nuclear parameters of normal and irradiated thymic lymphoid cells. These authors found that Cd had slight effects on the degree of DNA supercoiling in normal thymocytes. This effect was not concentration dependent. Hassoun & Stohs (1996) investigated the production of Cd-induced superoxide anions and nitric-oxide, DNA single strand breaks and the effects of Cd on LDH activity in cells of the macrophage cell line J774A.1. The authors observed a concentration dependant increase in DNA strand breaks. A maximum increase was seen at 0.4μM of Cd.

Richardson (1993) reports that Cd inhibits DNA synthesis *in vitro* in human peripheral blood lymphocytes and that it increases the frequency of chromatid breaks in mouse bone cells. According to Richardson & Gangolli (1994a), Pb is known to induce chromosomal aberrations and increases the frequency of sister chromatid exchange in mice bone marrow and human peripheral lymphocytes. In rodents, the micronucleus test, which is used to assess hazard after exposure (Anderson 1993), showed that Zn was genotoxic to the cells (Richardson & Gangolli 1994b).

According to Bryan (1981) Cd, Pb and Zn occur naturally within the cell. Of the three metals mentioned, only Zn is an essential metal that is required in trace concentrations. Zinc is localised in the cell nucleus and has an important role to play in DNA metabolism (Miller 1983) and it is associated with the catalytic activity of nuclear metalloenzymes (Bryan 1981).

Cadmium and Pb are toxic metals (Ratcliffe *et al.* 1993; Steffensen *et al.* 1994; Koropatnick & Zalups 1997). Lead in the nucleus is found within inclusion bodies and Cd is either bound to metallothionein proteins in the cytosol to high molecular weight proteins. When the trace concentrations of Zn, that are required for its normal metabolic functions in the cell are exceeded, Zn also becomes cytotoxic to the cell. Because the cell nucleus does not naturally sequester Zn, the excess Zn might actually cause damage to the DNA (and become genotoxic then) to which it has a metal ion association and is required to activate DNA synthesis (Bryan 1981).

Lead and Cd, as a result of their cytotoxicity to the cell nucleus, if found in excess in the cell nucleus, would be more easily sequestered than any excess Zn found in high concentrations within the cell nucleus. Cadmium however, only remains in the cell nucleus until equilibrium is reached and then it leaves the nucleus. This might explain why Cd has less of an effect on the DNA than does Pb. And also why Zn damages the DNA more than either Cd or Pb.

Chapter 6

Conclusions

Shrews from the immediate Stellenbosch region (i.e. a residential area in the town of Stellenbosch) were exposed to lower concentrations of Cd and Pb than were the shrews from the farming community of Raithby (situated on the outskirts of Stellenbosch). The results indicate that the levels of Cd found in the kidneys of the control shrews from both areas and also in the kidneys of the shrews that were experimentally exposed to Cd in the laboratory, are below the critical level of Cd quoted by Ma (1989) and Ma *et al.* (1991) that could result in adverse renal effects. Critical levels of Pb in the kidneys of the control and laboratory-exposed animals to Pb exceed the levels of Pb that Ma (1989) and Ma *et al.* (1991) deem critical in the kidneys of small mammals. The organ in which the highest levels of Cd and Pb were found was the bone. This was true for both the control shrews and the shrews that were exposed in the lab to these two metals.

Results on the activity of LDH as an indication of cell membrane integrity after exposure of mouse and shrew splenocytes to Cd, Pb and Zn, were inconclusive. This was attributed to the presence of heparin in the cell culture medium.

From the results of the DNA synthesis experiment measuring the rate of incorporation of $^{[3]}$ Thymidine into newly synthesised DNA in the whole blood cells of BALB/c mice, no trend could be observed. The results from the single shrew could not be used to make any conclusion since too few data were available.

Results of the cell viability experiments that employed the trypan blue dye exclusion technique indicated that Cd and Pb decreased the cell viability of the mouse cells and therefor adversely affected membrane integrity. The effects that Cd and Pb had on the integrity of the plasma membranes probably resulted from Cd and Pb changing the permeability of the membrane. The results of the experiment on Zn-exposed mouse cells *in vitro* showed that Zn increased the cell viability of the cells. This was probably because of Zn's ability to stabilise plasma membranes and to stimulate mitosis.

Cadmium, Pb and Zn were found to effect DNA damage in mouse whole blood cells that were exposed to the metals *in vitro* as indicated by measuring DNA strand breaks. The metals were found to have the following order of genotoxicity to the mouse whole blood cells: Cd<Pb<Zn.

It was found that different structures in the cell have different responses to the same metal. Metals may be more or less toxic to the cell depending upon where the effects of toxicity of the metals are measured in the cell. Cd, Pb and Zn do not effect the same degree of toxicity to plasma membrane integrity as they do to DNA integrity. The order of cytotoxicity of Cd, Pb and Zn, was the reverse order of genotoxicity of Cd, Pb and Zn to mouse whole blood cells that were exposed *in vitro* to the metals. Cadmium and Pb are more cytotoxic than Zn, while Zn is more genotoxic than either Cd or Pb. This is despite Cd and Pb being non-essential heavy metals and Zn being an essential trace metal.

From the results of the study, it can therefor be concluded that the shrew species in the Stellenbosch area might be under pressure due to fairly high concentrations of heavy metals in the environment. Further, it was shown that shrews readily take up Cd and Pb from their food (earthworms) and sequester it in different tissues and organs depending on exposure time.

It was also shown that the cell viability test using trypan blue exclusion and the comet assay measuring DNA damage are sensitive biomarkers of heavy metal toxicity on the cellular level.

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*Original not seen

Appendix 1

Comet Assay Solutions

Phosphate Buffered Saline (PBS: Ca^{2+}, Mg^{2+} free)

Per 1000ml dH_2O	
137mM NaCl	8g
2.7mM KCl	0.2g
4.3mM $Na_2HPO_4.12H_2O$	1.538g
1.4mM KH_2PO_4	0.2g
pH	7.3

Store at 4°C

1% Normal Melting Point Agarose (NMA)

NMA	500mg
PBS	50ml

0.5% Low Melting Point Agarose (LMPA)

LMPA	125mg
PBS	50ml

Store at 4°C

Stock Lysing Solution

Per 1000ml dH_2O	
2.5M NaCl	146.1g
100mM EDTA	37.2g
10mM Tris	1.2g

Add to ± 700ml dH_2O. Start to stir at low heat and add

Pellitised NaOH	12g

Stir till dissolved. Measure pH to 10.0 (Manipulate the pH of the solution with concentrated HCl or NaOH). QS to 890ml with dH_2O. Store at room temperature.

Working Lysing Solution

1% Triton-X	10ml
10% DMSO	100ml
Stock Lysing Solution	890ml

Stock Electrophoresis Buffer

10MNaOH	200g/500ml dH_2O
200mM EDTA	14.89g/200ml dH_20, pH 10.0

Store at 4°C

Working Electrophoresis Buffer

10MNaOH	30ml
200mM EDTA	5ml

QS to 1000ml with dH_20 and mix well

Neutralising Buffer

0.4M Tris	48.5g/800ml dH_20, pH 7.5

QS to 1000ml with dH_20, pH 7.5

Store at 4°C

Fluorescent Colouring Agent

Ethidiumbromide	20μg/ml

Store at 4°C

Appendix 2

Experimental Data

Weight(g) of Field Control Shrews (*Myosorex varius*)

	Raithby Control		Stellenbosch Control	
	Female	**Male**	**Female**	**Male**
Day 1	15.0	20.1	16.3	24.2
Day 2	10.9	15.1	15.54	24.5

Body Weight(g) of Laboratory Control Shrew (*Crocidura flavescens*)

	Raithby Control
	Female
Day 1	29.5
Day 2	
Day 3	25.9
Day 4	24.8
Day 5	23.6
Day 6	24.6
Day 7	23.8
Day 8	23.6
Day 9	24.0
Day 10	24.4
Day 11	25.3
Day 12	25.3
Day 13	26.6
Day 14	25.7
Day 15	25.4
Day 16	25.7
Day 17	25.1
Day 18	25.1

Weight(g) of Cadmium Exposed Shrews (*Myosorex varius*)

	Raithby Exposed		
	Female	**Female**	**Female**
Day 1	17.2	14.2	13.4
Day 2	14.5	14.2	12.3
Day 3	13.1	15.2*	12.2*
Day 4	15.4	13.3	12.1
Day 5	15.4	13.2	
Day 6	19.0*	13.3	
Day 7	15.3	13.9	
Day 8	16.1	14.0	
Day 9	14.8	13.3	
Day 10	14.1	14.2	
Day 11	13.9	14.7	
Day 12	13.9	14.8	
Day 13	13.8	15.1	
Day 14	13.2	14.6	
Day 15	13.2	14.9	
Day 16	13.6	14.7	
Day 17	13.7	14.0	
Day 18	14.7		
Day 19	14.4		
Day 20	14.2		
Day 21	15.1		
Day 22	16.1		
Day 23	16.2		
Day 24	16.0		
Day 25	16.7		
Day 26	16.9		
Day 27	15.4		

* Started to be fed with Cd contaminated earthworms (*Eisenia fetida*)

Weight(g) of Lead Exposed Shrews (*Myosorex varius*)

	Raithby Exposed	
	Female	**Male**
Day 1	16.4	17.2
Day 2	15.7	15.7
Day 3	14.5**	24.1**
Day 4	12.5	22.5
Day 5	23.2	14.4
Day 6	23.0	13.9
Day 7	13.9	13.7
Day 8	14.3	13.9
Day 9	13.2	13.3
Day 10	13.8	14.6
Day 11	14.5	14.2
Day 12	13.7	15.1
Day 13	13.4	14.9
Day 14	12.9	14.7
Day 15	13.4	14.3
Day 16	13.3	14.3
Day 17	13.1	14.4

** Started to be fed with Pb contaminated earthworms (*Eisenia fetida*)

Optical densities of BALB/c standards used to construct the standard curves

Sample ID	Optical density 1	LDH Units
Std 1	-219.3	0
Std 2	468	280.0
Std 3	624.7	640.0
Std 4	1100	1040.0
Std 5	1697	1530.0
Std 6	1820	2000.0

Optical densities of shrew standards used to construct the standard curves

Sample ID	Optical density 1	LDH Units
Std 1	-118.9	0
Std 2	268.4	280.0
Std 3	721.6	640.0
Std 4	1147	1040.0
Std 5	1624	1530.0
Std 6	1848	2000.0

Number of BALB/C Cells present in culture medium after the incubation period (E+07 or 08 represents the number of cells to the power ten)

Concentration	0µM	Cd 8µM	Pb 8µM	Zn 8µM
	9.83E+07	6.92E+07	5.67E+07	9.83E+07
	1.10E+08	7.02+07	5.33E+07	9.42E+07
	1.03E+08	6.92E+07	6.33E+07	8.92E+07
	8.17E+07	6.17E+07	5.75E+07	9.17E+07
	8.75E+07	5.50E+07	5.25E+07	8.17E+07
	1.12E+08	7.08E+07	5.67E+07	9.75E+07
	8.92E+07	7.42E+07	7.33E+07	8.42E+07
	8.50E+07	7.75E+07	5.92E+07	9.92E+07
Average	**9.58E+07**	**6.82E+07**	**5.91E+07**	**9.20E+07**

Number of BALB/C Cells present in culture medium after the incubation period (E+07 or 08 represents the number of cells to the power ten)

Concentration	0µM	Cd 16µM	Pb 16µM	Zn 16µM
	8.92E+07	6.92E+07	7.00E+07	8.00E+07
	9.00E+07	7.33E+07	6.42E+07	7.58E+07
	8.17E+07	7.25E+07	6.83E+07	9.00E+07
	7.50E+07	6.33E+07	7.25E+07	8.67E+07
	1.02E+08	7.25E+07	7.92E+07	8.83E+07
	8.42E+07	6.83E+07	7.08E+07	8.00E+07
	8.83E+07	6.58E+07	7.08E+07	8.00E+07
	8.50E+07	6.33E+07	5.92E+07	8.00E+07
Average	**8.69E+07**	**6.85E+07**	**6.94E+07**	**8.26E+07**

Number of BALB/C Cells present in culture medium after the incubation period (E+07 or 08 represents the number of cells to the power ten)

Concentration	0µM	Cd 20µM	Pb 20µM	Zn 20µM
	1.30E+08	9.33E+07	9.00E+07	1.18E+08
	1.27E+08	7.50E+07	8.67E+07	1.29E+08
	1.01E+08	6.58E+07	4.50E+07	8.92E+07
	1.03E+08	7.75E+07	7.17E+07	8.75E+07
	9.33E+07	5.17E+07	5.08E+07	8.25E+07
	1.02E+08	7.08E+07	6.92E+07	8.42E+07
	1.24E+08	7.42E+07	5.83E+07	9.75E+07
	8.42E+07	6.92E+07	6.25E+07	9.00E+07
Average	**1.08E+08**	**7.22E+07**	**6.68E+07**	**9.72E+07**

Number of BALB/C Cells present in culture medium after the incubation period (E+07 or 08 represents the number of cells to the power ten)

Concentration	0µM	Cd 40µM	Pb 40µM	Zn 40µM
	9.08E+07	7.75E+07	7.92E+07	1.06E+08
	1.20E+08	8.67E+07	6.92E+07	9.50E+07
	9.42E+07	7.00E+07	6.92E+07	8.92E+07
	1.07E+07	9.00E+07	6.50+E07	1.05E+08
	1.16E+08	1.05E+08	9.17E+07	1.10E+08
	1.19E+08	9.42E+07	8.50E+07	1.12E+08
	1.01E+08	9.00E+07	6.92E+07	9.92E+07
	6.17E+07	5.00E+07	5.83E+07	7.75E+07
Average	**8.92E+07**	**8.29E+07**	**7.45E+07**	**9.92E+07**

Number of BALB/C Cells present in culture medium after the incubation period (E+07 or 08 represents the number of cells to the power ten)

Concentration	0 µM	Cd 80µM	Pb 80µM	Zn 80µM
	1.00E+08	8.58E+07	8.33E+07	1.18E+08
	1.11E+08	8.83E+07	8.25E+07	1.02E+08
	9.00E+07	7.33E+07	7.33E+07	1.21E+08
	8.33E+07	7.42E+07	1.03E+08	1.15E+08
	1.02E+08	6.92E+07	7.17E+07	8.58E+07
	8.83E+07	8.08E+07	8.17E+07	1.11E+08
	1.27E+08	9.33E+07	9.00E+07	1.28E+08
	8.17E+07	**6.83E+07**	**6.58E+07**	**9.33E+07**
Average	**9.79E+07**	**7.92E+07**	**8.14E+07**	**1.09E+08**

Number of BALB/C Cells present in culture medium after the incubation period (E+07 or 08 represents the number of cells to the power ten)

Concentration	0 µM	Cd 100µM	Pb 100µM	Zn 100µM
	1.03E+08	7.00E+07	7.25E+07	1.13E+08
	1.09E+08	7.08E+07	7.50E+07	1.13E+08
	8.42E+07	6.33E+07	6.33E+07	1.11E+08
	8.92E+07	7.83E+07	5.67E+07	9.17E+07
	7.75E+07	6.50E+07	6.58E+07	8.75E+07
	7.58E+07	5.58E+07	5.67E+07	8.00E+07
	8.50E+07	5.75E+07	5.67E+07	9.67E+07
	7.67E+07	5.92E+07	5.67E+07	8.75E+07
Average	**8.76E+07**	**6.50E+07**	**6.29E+07**	**9.76E+07**

The number of cells present with comet tail lengths in a certain length interval

Interval	Class	nUV	pUV	20Cd	20Pb	20Zn	40Cd	40Pb	40Zn
0	0	679	136	208	330	366	386	300	313
5	5	129	61	92	54	1	14	14	0
10	10	94	3	0	8	6	0	45	4
15	15	20	0	0	1	3	0	8	38
20	20	60	0	0	7	8	0	21	37
25	25	0	0	0	0	1	0	0	0
30	30	12	0	0	0	15	0	11	8
35	35	0	0	0	0	0	0	0	0
40	40	6	0	0	0	0	0	0	0
45	45	0	0	0	0	0	0	0	0
50	50	0	0	0	0	0	0	0	0
55	55	0	0	0	0	0	0	0	0
60	60	0	0	0	0	0	0	1	0
Total #	Total #	1000	200	300	400	400	400	400	400
Interval	80Cd	80Pb	80Zn	100Cd	100Pb	100Zn	150Cd	150Pb	150Zn
0	216	312	100	362	360	8	128	288	201
5	63	6	126	29	12	217	221	1	65
10	9	33	92	9	19	142	34	85	88
15	5	6	15	0	1	19	8	8	14
20	3	34	19	0	4	9	8	7	28
25	2	9	39	0	0	0	0	0	0
30	2	0	7	0	4	5	1	11	3
35	0	0	0	0	0	0	0	0	0
40	0	0	2	0	0	0	0	0	1
45	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0
55	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0
Total #	300	400	400	400	400	400	400	400	400
Interval	200Cd	200Pb	200Zn	250Cd	250Pb	250Zn	500Cd	500Pb	500Zn
0	194	328	270	263	344	183	186	218	0
5	182	1	91	84	11	194	177	125	201
10	8	12	26	38	12	22	32	48	132
15	0	1	13	13	1	1	2	1	52
20	13	42	0	2	17	0	3	1	11
25	1	11	0	0	0	0	0	0	0
30	2	0	0	0	12	0	0	2	3
35	0	0	0	0	0	0	0	0	0
40	0	0	0	0	2	0	0	4	1
45	0	0	0	0	0	0	0	1	0
50	0	5	0	0	0	0	0	0	0
55	0	0	0	0	0	0	0	0	0
60	0	0	0	0	1	0	0	0	0
Total #	400	400	400	400	400	400	400	400	400

Example of a scoring work sheet for the comet assay

Comet Assay **Date_____**
Metal_____
Concentration_____
Slide #_____
